Remarks

Claims 1-13 were previously pending in the subject application. By this Amendment, Claims 1 and 2 have been amended and new claims 14-19 have been added. Accordingly, claims 1-19 are now before the examiner for consideration. In view of the remarks below, favorable consideration of the claims now presented is earnestly solicited.

The subject invention surprisingly and advantageously provides dried Factor VIII compositions which are stable even in the absence of a stabilizing amount of albumin. Blood factors, which are known to be delicate proteins, have for many years been obtained by extraction from serum. Those skilled in the art accept that blood factor compositions purified from serum will contain albumin because of the large quantity of albumin present in serum compared to the trace amounts of blood factors. When purifying compounds from blood it may generally be the goal to completely remove all components of the blood except for the target compound; however, in the case of delicate blood factors, it has been found to be desirable to leave albumin in the "purified" product in order to stabilize the factor.

That those in the art recognize the criticality of including albumin in Factor VIII compositions is evidenced by the current practice of adding albumin to purified Factor VIII. The practice of adding albumin to blood factors has continued despite the fact that albumin's origin as a blood component makes it less than ideal in, for example, pharmaceutical compositions.

Thus, it is quite unexpected, and highly advantageous, that the current applicant has found that the use of trehalose, even in the absence of albumin, is able to provide effective stabilization for dried Factor VIII. The applicant respectfully submits that the claims as now presented define an invention which was not previously known in the art and, in fact, runs directly counter to all of the relevant teachings at the time of the invention. Accordingly, favorable consideration of the claims now presented is earnestly solicited.

Claims 1 and 3 have been rejected under 35 U.S.C. §102(b) as being anticipated by Roser (BioPharm 4(8):47-53 (September 1991)) (Roser I). The applicant respectfully traverses this

grounds for rejection because the Roser I reference does not disclose or suggest a Factor VIII composition which is stabilized by trehalose in the absence of a stabilizing amount of albumin.

It is basic premise of patent law that, in order to anticipate, a single prior art reference must disclose within its four corners, each and every element of the claimed invention. In *Lindemann v. American Hoist and Derrick Co.*, 221 USPQ 481 (Fed. Cir. 1984), the court stated:

> Anticipation requires the presence in a single prior art reference, disclosure of each and every element of the claimed invention, arranged as in the claim. *Connell v. Sears Roebuck and Co.*, 722 F.2d 1542, 220 USPQ 193 (Fed. Cir. 1983); *SSIH Equip. S.A. v. USITC*, 718 F.2d 365, 216 USPQ 678 (Fed. Cir. 1983). In deciding the issue of anticipation, the [examiner] must identify the elements of the claims, determine their meaning in light of the specification and prosecution history, and identify corresponding elements disclosed in the allegedly anticipating reference. *SSIH, supra*; *Kalman [v. Kimberly-Clarke*, 713 F.2d 760, 218 USPQ 781 (Fed. Cir. 1983)] (emphasis added). 221 USPQ at 485.

In the current case, the claims specifically recite that the Factor VIII composition is stabilized with trehalose in the absence of a stabilizing amount of albumin. The absence of albumin is not disclosed explicitly or inherently by Roser I. Therefore, an anticipation rejection is improper.

As correctly noted by the Examiner, the Roser I reference is silent on the issue of whether albumin is present in the Factor VIII composition. Although the Office Action interprets Roser's silence on this issue as indicating an absence of albumin, the applicant respectfully submits that Roser's silence on this aspect of the composition should be interpreted as implying that the blood factor composition is prepared according to standard procedures known and followed by those skilled in the art. As discussed below, the skilled artisan would surely expect the presence of albumin in the Roser I Factor VIII composition. Thus, Roser I does not anticipate the current invention nor does Roser I provide any motivation for a person skilled in the art to dispense with the standard procedure of including albumin in Factor VIII compositions.

It is well established in the patent law that prior art references must be considered in the context of what they would teach those skilled in the art. In this regard the Federal Circuit has stated that additional references may be used to show how a person of ordinary skill in the art would have understood the teachings of the primary reference. *See Scripps Clinic & Research Foundation v.*

Genentech Inc., 927 F.2d at 1576, 18 U.S.P.Q.2d 1001, 1010 (Fed. Cir. 1991); *In re Donohue*, 632 F.2d 123, 125-26, 207 U.S.P.Q. 196, 199 (C.C.P.A. 1980); *In re Wiggins*, 488 F.2d 538, 179 U.S.P.Q. 421, 424 (C.C.P.A. 1970). The accompanying Exhibits clearly establish that, at the time of the subject invention, those skilled in the art accept that Factor VIII compositions, either purified or recombinant, contain albumin.

In order to provide insight into the state of the art with respect to blood factor compositions and the use of albumin to stabilize such compositions, the applicant is submitting herewith a number of Exhibits which establish that the standard practice of those skilled in the art is to stabilize Factor VIII compositions with albumin. The original filing date for the subject application was January 19, 1995. The applicant is submitting various Exhibits showing that, at the time of the subject invention, it was believed by those skilled in the art that, for highly labile Factor VIII, albumin was necessary as a stabilizer. The exhibits include the following:

1) Alpha Therapeutic Corporation advertisement in February 15, 2000 issue of the journal *Blood*:

> ...all licensed recombinant Factor VIII products contain albumin, which is necessary for preserving the factor proteins in recombinant products. (emphasis added).

2) Excerpt from 1999 Physicians Desk Reference describing the Helixate® recombinant Factor VIII product:

> The preparation is stabilized with albumin (Human) and *lyophilized.*

3) U.S. Patent No. 4,361,509 describing the stabilization of purified porcine Factor VIII:

> Preparations of VIII: c obtained from a porcine plasma source should be stabilized within 5 to 10% human serum albumin prior to storage.
> Column 10, lines 1-3.

4) Expert Delcaration of Dr. Alan Mackenzie:

> Factor VIII preparations which were derived from human blood necessarily contained albumin. Although there were significant health risks associated with administering albumin to a patient, namely the potential risk for viral contaminants to be present, the presence of albumin was believed to be necessary in order to stabilise the Factor

> VIII protein. Recombinant Factor VIII preparations were also being prepared, but again it was believed to be necessary to add albumin to the preparations to stabilise the proteins. The presence of albumin was believed to be necessary because Factor VIII proteins are extremely labile, even in the presence of other stabilisers.

The applicant is also submitting herewith the following additional Exhibits which further establish that, at the time of the subject invention (and even years later), those skilled in the art believed that albumin was necessary to stabilize Factor VIII preparations:

A. The current University of North Carolina Hemophilia Center Web Site (www.med.unc.edu):

> Monoclonal antibody purified Factor VIII. Prepared from pooled human plasma which is screened for anti-HIV 1 and 2, anti-HBc, ALT, anti-HTLV I/II, and anti-HCV. The Factor VIII is purified by affinity chromatography using mouse monclonal antibodies to human Factor VIII. The purified Factor VIII prior to formulation has a specific activity of ~3000 units per mg. Human albumin is used as a stabilizer in the formulation of the Factor VIII.
>
> . . .
>
> Recombinant Factor VIII. Recombinant Factor VIII is a synthetic form of Factor VIII prepared in mammalian cells, such as Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells. Because both products are formulated with human albumin, even though the albumin is pasturized, recombinant Factor VIII is subject to the same recalls as plasma-derived Factor VIII in the event that donors with Creutzfeld-Jacob disease contribute to the source plasma from which the albumin is prepared. Two forms of recombinant Factor VIII are currently licensed for use. (emphasis added)

B. Brownlee *et al.* article:

> Both recombinant Factor VIII products (Genetics Institute of Genentech), after purification by immuno-affinity chromatography with monoclonal antibodies, ion-exchange chromatography and other methods, were reported to be essentially indistinguishable from plasma-derived Factor VIII [45, 48]. The full-length recombinant Factor VIII produced by both companies may be regarded as a "first-generation" product, since the CHO cells used to produce Factor VIII were grown in a tissue culture medium that contained protein additives. Moreover, the resultant purified Factor VIII was stabilized by the addition of human albumin. (emphasis added)

C. Puget Sound Blood Center Web page (www.psbc.org; revised November 1999)

Concentrates differ in the purification procedures. Highly purified Factor VIII, e.g. preparations purified over a monoclonal antibody column or current recombinant Factor VIII concentrates, are stabilized by adding 98% of pasteurized human albumin. (emphasis added)

D. Emory Health Sciences Press Release (www.emory.edu; October 10, 2000)

In the early 1990s, scientist carried safety one step further with genetically engineered recombinant Factor VIII products made by inserting the Factor VIII gene into a cell line and producing mass quantities of concentrated human Factor VIII. Although these products contained no human or animal products, they were stabilized with a small amount of albumin, a human blood component. (emphasis added)

E. National Hemophilia Foundation Website (www.hemophilia.org; January 10, 2001)

ReFacto is the first recombinant Factor VIII product formulated without human serum albumin in its final formulation. (emphasis added)

F. U.S. Patent No. 6,171,825 (filed September 4, 1998)

For labile proteins such as Factor VIII, human albumin has been added as a stabilizer during the preparation and purification procedures. Although the albumin is subjected to a viral inactivation step by pasteurization, it would be ideal if recombinant Factor VIII could be manufactured in the complete absence of human and animal blood proteins.

G. WO 94/07510 (published April 14, 1994)

A formulation with a low amount of protein will generally lose activity during purification, sterile manufacturing, in the package and during the administration. This problem is usually solved by the addition of human albumin which reduces the activity loss of the active protein considerably. Human albumin functions as a general stabilizer during purification, sterile manufacturing and freeze-drying (see review by Wang *et al.*, *J. of Parenteral Sci.* and Tech. Vol. 42, Number 2S, supplement. 1988). Human albumin is also a good cake-former in a formulation for freeze-drying. The use of albumin for stabilization of Factor VIII is known and is currently used in all highly purified Factor VIII products on the market. (emphasis added)

However, it is not desirable to add human albumin to a therapeutic protein manufactured by recombinant DNA technology. In addition, the use of human albumin as a formulation excipient often limits the use of many of the most powerful and sensitive analytical methods for protein characterization.

> There is a need for albumin free formulations containing Factor VIII and especially recombinant Factor VIII which are stable during drying or freeze-drying, in solution and as a solution after reconstitution.

It is apparent from the accompanying Exhibits that, at the time of the subject invention, it was widely accepted that human albumin was a necessary evil for stabilizing Factor VIII. Furthermore, to the extent that other stabilizing agents had been utilized as a substitute for albumin, these alternative stabilizers were a select few and did not include trehalose. For example, the formulation described in WO 94/07510 (Exhibit G) contains a non-ionic surfactant, such as a block copolymer, that functions to stabilize Factor VIII. Example 1 at page 10 of WO 94/07510 shows that, following reconstitution, the recovery of Factor VIII: C with non-ionic surfactant was equivalent to that with albumin. Thus, it was highly surprising and unexpected for the current applicant to demonstrate stability of Factor VIII in excess of 95% after four weeks (specification, Example 2) when Factor VIII preparations are freeze-dried using trehalose as a stabilizer in the absence of albumin. Factor VIII compositions prepared in accordance with the method of the subject invention advantageously retain their activity for four weeks or more (specification, Example 2). Such an excellent stabilizing effect was highly unexpected in view of the widely-held belief of those skilled in the art that labile Factor VIII needed albumin to be stabilized.

There is no question that the Roser I reference does not explicitly state whether albumin is present. There is also no question that the absence of albumin is an express limitation in the applicant's claims. The Office Action appears to take the categorical position that the absence of an express statement that an ingredient is present equates to having an express statement that the ingredient is absent. This is incorrect as a matter of logic, common sense, and law.

The Office Action provides absolutely no basis for the bold proposition that the absence of any mention of an ingredient is the same as an express statement that the ingredient is not present. The applicant believes that no basis for this premise exists. Therefore, the applicant respectfully requests a specific identification by the Examiner of the basis for the quite remarkable proposition that the absence of any mention of an ingredient equates to an express statement that the ingredient is

not present.

Because there is no logical basis for stating that, in every instance, the absence of any statement equals an express statement that something is not present, it is necessary to examine the facts in each case before drawing a conclusion. For example, if I say that "I drank a cup of tea" it may well be reasonable to conclude that there was not any cyanide in my tea. However, my statement says nothing about whether there was sugar, milk, or honey in my tea. Furthermore, it would surely not be reasonable to conclude (based on my statement) that there was no water in my tea (even though I did not mention any water). Thus, common sense, practical experience, and informed knowledge of the subject matter are all helpful in trying to determine whether 1) the absence of an explicit statement about an ingredient indicates that the ingredient was absent, 2) that the absence of an explicit statement says nothing about the likely presence or absence of the ingredient, or 3) that the absence of any statement suggests that the ingredient is present because that ingredient is virtually always present and its unusual absence would surely be noted.

In applying these principles to Patent Law and, specifically, to the current case, we must look to the law of inherency. The Examiner admits that Roser I is silent on the issue of whether albumin is present. It is equally undeniable that the applicant's claims explicitly recite the absence of a stabilizing amount of albumin as a claim limitation. Thus, the Roser I reference does not disclose one of the applicant's claim limitations. The applicant concedes that a claim may be anticipated even if a single prior art reference does not explicitly disclose all of the limitations of the claimed invention if, and only if, any omitted limitations are inherent in the cited reference. Thus, the current Office Action is, in effect, stating that the absence of albumin is "inherent" in the Roser I reference.

Unfortunately, the Office Action falls far short of meeting the legal requirements for basing an anticipation rejection on inherency. Under the Patent Laws, a prior art rejection based on inherency is only proper if the prior art necessarily resulted in the claimed subject matter. *In re King*, 801 F2d 1324, 1326, 231 USPQ 136, 138 (Fed. Cir. 1986). Further,

> the doctrine of inherency is available only when the prior inherent event can be established as a certainty. That an event may result from a given set of circumstances is not sufficient to establish anticipation. . . . A prior inherent

> event cannot be established based on speculation, or where a doubt exists (emphasis added). *Ethyl Molded Product Co. v. Betts Package Inc.*, 9 USPQ 2d 1001, 1032-33 (E.D. KY 1988).

In the current case it cannot reasonably be stated that albumin was necessarily absent from the Roser I composition. In fact, quite the contrary is true; one skilled in the art would expect that albumin was present. As discussed above, the applicant has submitted extensive materials establishing that, at the time of the invention, it was accepted by those skilled in the art that albumin was necessary to stabilize Factor VIII.

The purpose of 35 U.S.C. §102 is to prevent the granting of a patent which would remove from the public something which has already been placed in the public domain. In *Dewey & Almy Chem. Co. v. Mimex Co.*, Judge Learned Hand wrote:

> No doctrine of the patent law is better established than that a prior patent . . . to be an anticipation must bear within its four corners adequate directions for the practice [of the subsequent invention] . . . if the earlier disclosure offers no more than a starting point . . . if it does not inform the art without more how to practice the new invention, it has not correspondingly enriched the store of common knowledge, and it is not an anticipation. 124 F.2d 986, 990; 52 USPQ 138 (2nd Cir. 1942).

In the current case it is readily apparent from the foregoing remarks and the accompanying Exhibits that, at the time of the subject invention, the public was not in possession of albumin-free Factor VIII compositions or methods for making such compositions. Accordingly, the applicants respectfully request reconsideration and withdrawal of the rejection under 35 U.S.C. §102(b) based on Roser I.

Claims 1-13 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Roser (Roser I) and Livesey *et al.* (U.S. Patent No. 5,364,756) in view of Roser (U.S. Patent No. 4,891,319, Roser II) and Lee *et al.* (EP 0 314 095). The applicant respectfully traverses this grounds for rejection because the cited references, alone, or in combination, do not disclose or suggest an albumin-free dried Factor VIII composition stabilized with trehalose.

Read in the context of what was known in the art at the time, the Livesey *et al.* disclosure does not teach or suggest the applicant's unique and advantageous Factor VIII compositions or their

methods of preparation. A fair reading of Livesey *et al.* reveals that this reference pertains only tangentially to Factor VIII compositions. To the extent that Livesey *et al.* address the preparation of Factor VIII compositions at all, Livesey *et al.* only reinforce what was accepted in the art at that time — that albumin was necessary in order to prepare a stable Factor VIII composition.

Livesey *et al.* provide a method and apparatus for cryopreserving and drying various biological materials. The emphasis of the Livesey *et al.* reference is on a unique apparatus, rather than sample preparation. In fact, Livesey *et al.* state that "[t]he exact ingredients of each available suspension is not considered to be a component of this invention." (emphasis added).

To provide further insight into the state of the art at the time of the subject invention, the applicant requested Dr. Alan Mackenzie, a renowned expert in this field to comment on the disclosure of the cited Livesey *et al.* reference and what that disclosure would teach one skilled in the art. In an accompanying Expert Declaration Dr. Mackenzie states:

> My knowledge in this regard is based on many years of experience in this field dating back to 1959. In 1996, various Factor VIII compositions were known. Factor VIII preparations which were derived from human blood necessarily contained albumin. Although there were significant health risks associated with administering albumin to a patient, namely the potential risk for viral contaminants to be present, the presence of albumin was believed to be necessary in order to stabilize the Factor VIII protein. Recombinant Factor VIII preparations were also being prepared, but again it was believed to be necessary to add albumin to the preparations to stabilize the proteins. The presence of albumin was believed to be necessary because Factor VIII proteins are extremely labile, even in the presence of other stabilizers.

In evaluating the patentability of an invention, it is critical to review a cited reference for what it would have taught one skilled in the art. The Livesey *et al.* reference does not suggest that trehalose could be used, in the absence of albumin, to stabilize Factor VIII. Rather, Livesey *et al.* clearly teach that albumin can be used in combination with trehalose.

As noted by Dr. Mackenzie in the accompanying Expert Declaration:

> [t]rehalose is only mentioned by Livesey *et al.* as one possible ingredient in a drying process. There is no reference to preparing a Factor VIII composition in the absence of albumin, and it cannot be inferred, given the knowledge at that time, that albumin

> was not required. Thus, the Livesey *et al.* discussion of stabilising agents is entirely consistent with the proposition that Factor VIII cannot be freeze-dried without the use of albumin as a stabilising agent.

In addition to its lack of any new teaching with regard to sample preparation, the relevance of the Livesey *et al.* patent to freeze-drying Factor VIII is further diminished due to the focus of Livesey *et al.* on whole cells, rather than proteins. In this regard, Dr. Mackenzie observes that:

> [r]ed blood cells, plateletes, leukocytes, sperm, pancreatic islets, and marrow cells are all listed as specific examples of cells which can be preserved using the Livesey *et al.* reference. There is very little discussion, and there are no examples, of the preservation of proteins. Those skilled in the art know that materials and procedures used to preserve whole cells and/or viruses are not necessarily applicable to the stabilisation of proteins. Furthermore, Livesey *et al.* state that the exact ingredients of the suspensions which are to be preserved "is not considered to be a component of the invention." Thus, I find no disclosure in the Livesey *et al.* patent which is specifically relevant to the selection of appropriate stabilising agents for delicate proteins.

Thus, as with the Roser I reference discussed above, the Livesey *et al.* reference is silent on the issue of whether albumin would be present in the Factor VIII compositions. In interpreting the teachings of Livesey *et al.*, it must be acknowledged that the main focus of this reference is on a cryopreservation method. The Livesey *et al.* reference only mentions Factor VIII briefly and certainly provides no teaching which would lead a person skilled in the art to depart from the well-accepted standard procedure of stabilizing blood factors with albumin. In view of the fact that the need for a stabilizing agent is particularly acute when the blood factor is going to be subjected to further processing such as drying, the applicant respectfully submits that the Livesey *et al.* disclosure provides no motivation to remove albumin as a component of Factor VIII compositions.

As noted by the Court of Appeals for Federal Circuit, the specification, of which the claims are part, teaches about the problems solved by the claimed invention, the way the claimed invention solves these problems, and the prior art that relates to the invention. These teachings provide valuable context for the meaning of the claim language. *Eastman Kodak Co., v. the Goodyear Tire & Rubber Co.*, 42 USPQ 2d 1737, 1741 (Fed. Cir. 1997). It is the person of ordinary skill in the field

of the invention through whose eyes the claims are construed. Such person is deemed to read the words used in the patent documents with an understanding of their meaning in the field, and to have knowledge of any special meaning and usage in the field. The inventor's words that are used to describe the invention—the inventor's lexicography—must be understood and interpreted by the court as they would be understood and interpreted by a person in that field of technology. *Multiform Desiccants, Inc. v. Medzam, Ltd.*, 45 USPQ 2d 1429, 1432 (Fed. Cir. 1998).

The teachings of Livesey *et al.* must be considered for what they taught a person skilled in the art who was already aware of the long-standing and widely-accepted practice of using albumin to stabilize Factor VIII. When read in this light, the teachings of Livesey *et al.* do not teach the skilled artisan that stable Factor VIII preparations can be prepared using trehalose in the absence of albumin.

The shortcomings of the primary references have been discussed above. The Lee *et al.* reference and Roser II do not cure the defects of the primary references. The Roser II patent describes the addition of trehalose to blood components (antibodies) obtained directly from serum. Such a serum preparation would most certainly contain albumin. Thus, there is no teaching in this reference which would lead a person skilled in the art to depart from the standard and accepted practice of using albumin to stabilize Factor VIII compositions.

Although Lee *et al.* discusses the use of calcium chloride, there is no indication that albumin is not necessary in the composition. Specifically, these references provide no teaching or motivation for the skilled artisan to depart from the standard practice of using albumin to stabilize blood factors.

The mere fact that the purported prior art could have been modified or applied in manner to yield applicant's invention would not have made the modification or application obvious unless the prior art suggested the desirability of the modification. *In re Gordon*, 221 U.S.P.Q. 1125, 1127 (Fed. Cir. 1984). Moreover, as expressed by the CAFC, to support a §103 rejection, "[b]oth the suggestion and the expectation of success must be founded in the prior art . . . " *In re Dow Chemical Co.*, 837 F.2d 469, 473, 5 U.S.P.Q. 2d 1529, 1531 (Fed. Cir. 1988). In the references cited in support of the §103 rejection, one finds neither.

As noted above, prior art references must be viewed in the context of what they would teach a person skilled in the art. See *Joy Technologies Inc. v. Quigg*, 732 F. Supp. 227, 232-33, 14 U.S.P.Q.2d 1432, 1438 (D.D.C. 1990). In determining what Lee *et al.* would teach a person skilled in the art, please note that Lee *et al.* refer to U.S. Patent No. 4,361,509 as providing appropriate teachings for obtaining "highly purified" Factor VIII. See page 2, lines 35-38. As explained above, Lee *et al.* teach that the Factor VIII obtained after purification should be stabilized with albumin. Thus the teachings of Lee *et al.* (and the '509 patent) are entirely consistent with the proposition that the art accepted albumin as a necessity for stabilizing Factor VIII. Thus, Lee *et al.*, the '509 patent and the other identified art actually teach away from the current invention which surprisingly and advantageously provides a stabilized dried Factor VIII composition without albumin.

In view of the wide-spread belief at the time of the invention that albumin was necessary to stabilize labile Factor VIII it was both very unexpected and highly advantageous to find that Factor VIII can be effectively stabilized with trehalose in the absence of albumin. Thus, this invention unexpectedly satisfies a well-documented long-felt need. Accordingly, the applicant respectfully requests reconsideration and withdrawal of the rejection under 35 U.S.C. §103 based on Roser I and Livesey *et al.* in view of Roser II and Lee *et al.*

Please note that the applicant is submitting herewith a copy of Curtis *et al.* U.S. Patent No. 5,576,291, which has been cited in a related application. The applicant respectfully submits that the Curtis *et al.* reference does not anticipate or render obvious the applicant's claims.

In considering the relevance of the Curtis *et al.* reference, it should be noted that Factor VIII and activated Factor VIII (Factor VIIIa) are different entities. Factor VIII is produced as a protein of 2332 amino acids. Factor VIII is converted to activated Factor VIII (Factor VIIIa) through a multistep enzymatic process. Indeed, Curtis *et al.* clarifies the definition by stating that the "activated" form is a product of the "conversion of the Factor VIII protein molecule" (col. 2, lines 48-52). Despite the similarity of the names of "Factor VIII" and "Factor VIIIa", these entities have very different chemical, physical, and physiological properties. As described by Dr. Preston in the accompanying Expert Declaration under 35 USC 1.132, those skilled in the art are very familiar with

the nomenclature whereby "Factor VIII" refers to the labile, non-activated protein, whereas "Factor VIIIa" refers to the heterotrimer formed after substantial chemical modification occurs to Factor VIII.

The Curtis *et al.* reference provides a method for treating patients who have developed inhibitors to Factor VIII. Thus, for these patients, administered Factor VIII is unable to function properly in the cascade of physiological events leading to blood clotting. Curtis *et al.* found that individuals with this condition can be treated by administering activated Factor VIII. The very fact that activated Factor VIII is effective when Factor VIII is not underscores the fundamental differences between these two compounds.

Thus, it is apparent that 1) Factor VIIIa is recognized by those skilled in the art as a distinct and separate entity from Factor VIII, 2) that the properties of Factor VIIIa are very different from the properties of relatively unstable Factor VIII.

The Curtis *et al.* reference pertains to activated Factor VIII. In view of the aforementioned differences between activated Factor VIII and native Factor VIII, the applicant respectfully submits that those skilled in the art would not consider stabilization techniques used for Factor VIIIa to be necessarily applicable to Factor VIII.

The applicant further respectfully submits that, to the extent that the Curtis *et al.* teachings with regard to stabilization of Factor VIIIa are discernable, it appears that they focus on factors other than the use of trehalose. For example, Curtis *et al.* refer to "maintaining the concentration of the activated human Factor VIII at a stabilizing level" (column 2, lines 44-46). Where Curtis *et al.* describe stabilization procedures in any detail (column 5 lines 32-65) there is no indication that even activated Factor VIII could be freeze-dried with trehalose in the absence of albumin. Thus, although trehalose is listed among several stabilizers that can be used (as is albumin), one of ordinary skill in the art would not conclude that trehalose can be used in the absence of a stabilizing amount of albumin, even for activated Factor VIII.

Accordingly, the applicant respectfully submits that Curtis *et al.* do not teach or suggest the use of trehalose, in the absence of albumin, to stabilize highly labile Factor VIII.

Claims 1-13 have been rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over the claims of U.S. Patent No. 4,891,319 (Roser II), in view of Roser (*BioPharm* 4(8):47-53, September 1991, (Roser I)) and Livesey *et al.* (U.S. Patent No. 5,364,756) and Lee *et al.* (EP 0 314 095). The applicant respectfully traverses this grounds for rejection because the claims of Roser II, alone or in combination with the secondary references, do not make obvious the currently-claimed invention. As discussed above, nothing in the cited references teaches or even suggests that dried stabilized Factor VIII could be prepared in the absence of albumin. In the absence of such a teaching or motivation founded in the prior art, a finding of obviousness is improper. Therefore, the applicant respectfully requests reconsideration and withdrawal of the obviousness-type double patenting rejection.

Claims 1-13 have been provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claim 7 of copending Application No. 08/875,796, in view of Roser (*BioPharm* 4(8):47-53, September 1991, (Roser I)) and Livesey *et al.* (U.S. Patent No. 5,364,756) and Lee *et al.* (EP 0 314 095). Upon an indication of allowability the applicant will submit a Terminal Disclaimer with respect to the co-pending application.

Conclusion

Factor VIII is essential for the treatment of haemophilia. Unfortunately, Factor VIII is extremely labile, and, therefore, it is difficult to produce compositions which contain stable Factor VIII. The subject invention provides a unique, advantageous, and unexpected method for preparing stable freeze-dried Factor VIII.

Haemophilia is a coagulation disorder which affects about 1 in every 10,000 males. Haemophilia A is the most common form of hemophilia and is due to a deficiency in Factor VIII. Most instances of hemophilia are associated with a genetic mutation that causes the Factor VIII deficiency. A relatively small percentage of patients with hemophilia A develop inhibitors to Factor VIII. For years, the main treatment for bleeding episodes was fresh frozen plasma or cryoprecipitate. Eventually, Factor VIII was purified from pooling of blood donations. Although recombinant Factor

VIII has been available since the mid-1980's, until recently all commercial Factor VIII preparations were stabilized with human albumin because those skilled in the art believed that albumin was necessary to stabilize the extremely labile Factor VIII. The use of albumin as a stabilizing agent leaves open the possibility for contamination of Factor VIII preparations. Thus, there is a long-felt need for a method of stabilizing Factor VIII without albumin. The current invention satisfies that long-felt need.

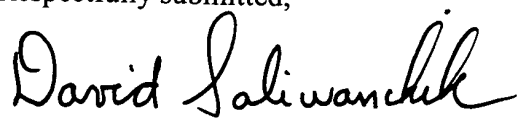
Advantageously, the current applicant's invention provides unique and advantageous Factor VIII compositions. The methods and products of the subject invention are particularly advantageous because of the absence of albumin. Prior to the applicant's invention, it was generally accepted by those skilled in the art that albumin was necessary as a stabilizer when drying Factor VIII. The applicant respectfully requests favorable consideration of the pending claims in view of this important contribution to the art.

In view of the foregoing remarks and the amendments above, the applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

The applicants also invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephone interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



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DRS/la
Attachment: Petition and Fee for Extension of Time in triplicate
Marked-up Version of Amended Claims
Amendment Transmittal Form in triplicate
Exhibits 1-4
Exhibits A-G
Declaration of Dr. Preston
Supplemental Information Disclosure Statement
Form PTO-1449
Copy of U.S. Patent No. 5,576,291

Marked-up Version of Substituted Claims:

Claim 1 (amended):

A stable dried Factor VIII composition containing a stabilizing amount of trehalose in the absence of a stabilizing amount of albumin.

Claim 2 (amended):

The composition, according to claim 1, containing 0.15 to2.5 mg trehalose per unit of [blood factor] Factor VIII.

EXHIBIT 1

blood

JOURNAL OF
THE AMERICAN
SOCIETY OF
HEMATOLOGY

VOLUME 95
NUMBER 4
FEBRUARY 15, 2000

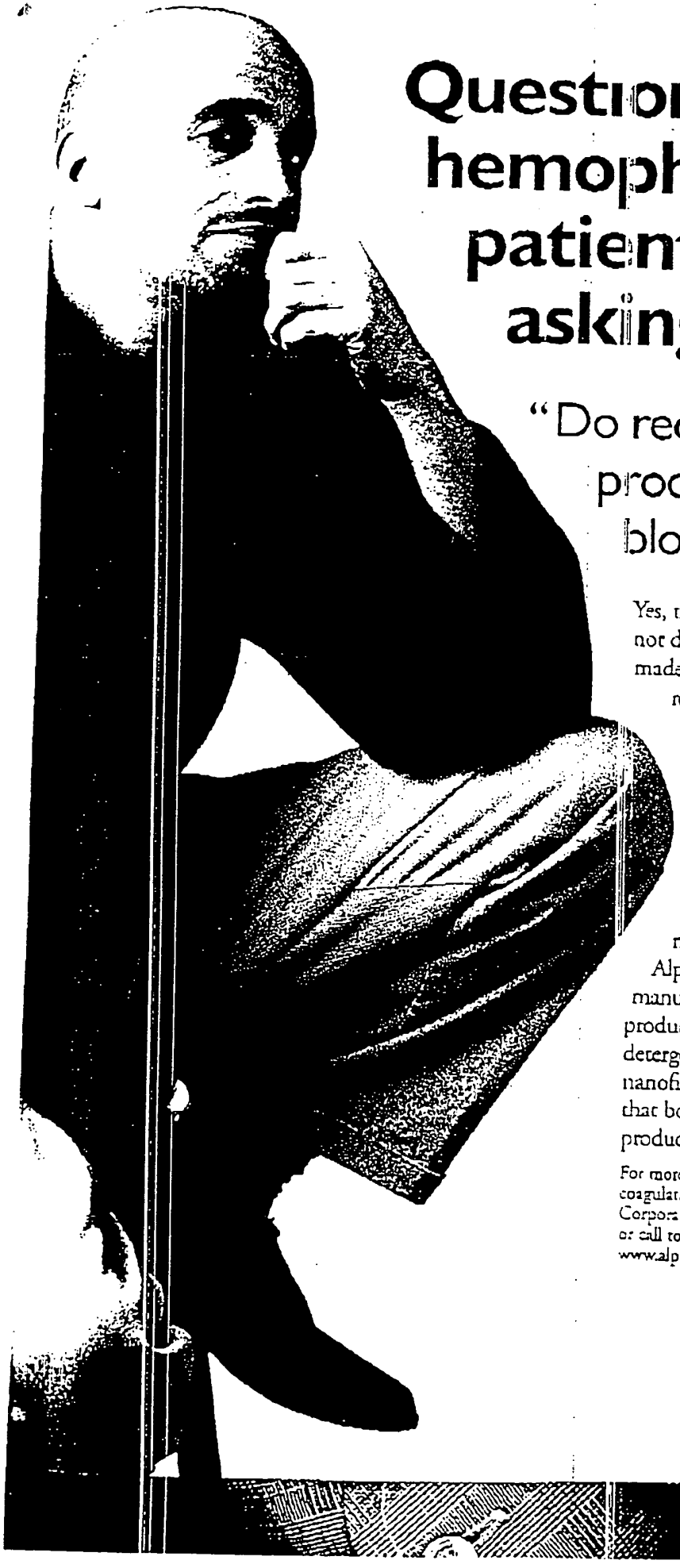
Natural anticoagulants and sepsis

Sickle cell day hospitals

PCR detection of haptoglobin anaphylaxis

P53 and CML blast crisis

Blood 15/2/00 issue



Questions hemophilia patients are asking:

"Do recombinant Factor VIII products contain human blood elements?"

Yes, they do. The factor proteins themselves are not derived from human blood. They are made from animal cells. However, all licensed recombinant Factor VIII products contain albumin, which is necessary for preserving the factor proteins in recombinant factor products. Albumin is derived from pooled human plasma, much the same way as plasma-derived factor products.

Today there are many effective safeguards against viral contamination of products made from human plasma. Safeguards Alpha Therapeutic Corporation® uses in manufacturing its plasma-derived factor products include affinity chromatography, solvent detergent treatment, heat treatment and nanofiltration. These kinds of safeguards mean that both plasma-derived and recombinant factor products are very safe and effective.

For more information about Alpha Therapeutic Corporation® coagulation factor products, write to: Alpha Therapeutic Corporation, 5555 Valley Boulevard, Los Angeles, CA 90032 or call toll free: 1 (800) 292-6118 or visit our web site at www.alphather.com.

alpha®
THERAPEUTIC CORPORATION

EXHIBIT 2

Gammar-P I.V.—Cont.

10) Allow the product vial to remain undisturbed for 5 minutes after diluent addition. Do not touch or mix during this time.

11) After 5 minutes, mix the product vial by gently swirling the vial without creating excessive foam. Never shake the product vial.

Note: A syrup-like layer may remain on the bottom of the vial following reconstitution. Swirl gently to disperse this layer until a homogenous solution is obtained.

12) Examine solution. All unreconstituted product should dissolve with gentle swirling and the solution should be clear and ready to administer in 20 minutes or less.

13) Product contains no preservative. Infusion must be initiated within 3 hours of reconstitution. If not used within this time frame, it should be properly disposed of and not administered.

14) Reconstituted product does not need to be filtered. If a filter is used, it should be a 15 micron filter or larger.

15) If several doses of Immune Globulin Intravenous (Human), Gammar®-P I.V., are to be pooled aseptically for administration, avoid excessive formation of foam in the pooling container and gently swirl the pooling container to mix. **DO NOT SHAKE THE POOLING CONTAINER.**

Administration

CAUTION: When entering the product stopper with an IV set spike for administration, care should be taken to follow the path made by the transfer spike (see Reconstitution).

Immune Globulin Intravenous (Human), Gammar®-P I.V., is to be administered by intravenous infusion. The infusion should begin at a rate of 0.01 mL/Kg/minute, increasing to 0.02 mL/Kg/minute after 15 to 30 minutes. Most patients tolerate a gradual increase to 0.03 – 0.06 mL/Kg/minute. For the average 70 kg person this is equivalent to 2 to 4 mL/minute. If adverse reactions develop, slowing the infusion rate will usually eliminate the reaction. Discard any unused solution.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration whenever solution and container permit.

HOW SUPPLIED

Individual Vial Packages

Immune Globulin Intravenous (Human), Gammar®-P I.V., is supplied in single dose vials, with diluent and sterile, vented transfer spike for reconstitution. The 10 g dosage form package also contains an administration set. The following dosage forms are available:

[See table at top of previous page]

Bulk Package

Immune Globulin Intravenous (Human), Gammar®-P I.V., 5 g immune globulin/vial is supplied in a bulk pack (NDC 0053-7486-06) of six (6) single dose vials. Each single dose vial should be reconstituted with 100 mL Sterile Water for Injection, U.S.P. (not supplied).

STORAGE

When stored at temperatures not exceeding 25°C (77°F), Immune Globulin Intravenous (Human), Gammar®-P I.V., is stable for the period indicated by the expiration date on its label. Avoid freezing which may damage container for the diluent.

CAUTION: FEDERAL LAW PROHIBITS DISPENSING WITHOUT PRESCRIPTION.

REFERENCES

1. Data on File: Armour Pharmaceutical Company.
2. Steele RW, Augustine RA, Tannenbaum AS, Marmer DJ. Intravenous Immune Globulin for Hypogammaglobulinemia: A Comparison of Opsonizing Capacity in Recipient Sera. *Clin. Immunol. Immunopathol.* 1985; 34:275–283.
3. Martindale. *The Extra Pharmacopoeia* 27th ed. Edited by Wade A. London: The Pharmaceutical Press. 1979; 65.
4. Fudenberg HH. Sensitization to Immunoglobulins and Hazards of Gamma Globulin Therapy. *Immunoglobulins. Biologic Aspects and Clinical Uses.* 1970; 211–220. Edited by Merler E. National Academy of Sciences, Washington, D.C.
5. Sekul EA, Cupler EJ, Dalakas, MC. Aseptic Meningitis Associated with High Dose Intravenous Immunoglobulin Therapy: Frequency and Risk Factors. *Ann Int Med* 1994; 121:4, 259–262.
6. Kato E, Shindo S, Eto Y, et al. Administration of Immune Globulin Associated with Aseptic Meningitis. *JAMA* 1988; 259:3269–3271.
7. Casteels-Van Daele M, Wijndaele L, Hunninck K, Gillis P, Ziekenhuis V. Intravenous Immune Globulin and Acute Aseptic Meningitis. *N Engl J Med* 1990; 323:614–615.
8. Scribner C, Kapit R, Phillips E, Rickels N. Aseptic Meningitis and Intravenous Immunoglobulin Therapy. *Ann Int Med* 1994; 121:305–306.
9. Tan E, et al. Acute Renal Failure Resulting from Intravenous Immunoglobulin Therapy. *Arch Neurol.* 1993; 50(2): 137–139.

BIBLIOGRAPHY

Polley MJ, Fischetti VA, Landaburu PH. Native Intravenous IgG Exhibits Greater Biological Activity than Modified IgG. From the *XV Cong. Int. Soc. of Hematology*: 1984.

Revised: June, 1997 IBM 12173

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Antihemophilic Factor (Recombinant)
HELIXATE® ℞

DESCRIPTION

Antihemophilic Factor (Recombinant), HELIXATE® is a sterile, stable, purified, non-pyrogenic, dried concentrate which has been manufactured by recombinant DNA technology. HELIXATE is intended for use in therapy of classical hemophilia (hemophilia A). HELIXATE is produced by Baby Hamster Kidney (BHK) cells into which the human factor VIII (FVIII) gene has been introduced.[1] HELIXATE is a highly purified glycoprotein consisting of multiple peptides including an 80 kD and various extensions of the 90 kD subunit. It has the same biological activity as FVIII derived from human plasma. In addition to the use of the classical purification methods of ion exchange chromatography and size exclusion chromatography, monoclonal antibody immunoaffinity chromatography is utilized along with other steps designed to purify recombinant factor VIII (rAHF) and remove contaminating substances. The final preparation is stabilized with Albumin (Human) and lyophilized. The concentration of HELIXATE is approximately 100 IU/mL. The product contains no preservatives.

Each vial of HELIXATE contains the labeled amount of rAHF in international units (IU). One IU, as defined by the World Health Organization standard for blood coagulation factor VIII, human, is approximately equal to the level of factor VIII activity found in 1.0 mL of fresh pooled human plasma. The final product when reconstituted as directed contains the following excipients: 10–30 mg glycine/mL, not more than (NMT) 500 µg imidazole/1000 IU, NMT 600 µg polysorbate 80/1000 IU, 2–5 mM calcium chloride, 100–130 mEq/L sodium, 100–130 mEq/L chloride, and 4–10 mg Albumin (Human)/mL. HELIXATE must be administered by the intravenous route.

CLINICAL PHARMACOLOGY

The clinical trial of HELIXATE has included 168 patients, enrolled over a 55-month period. A total of 16,186 infusions have been utilized in this trial. The study was conducted in several stages.

Initial pharmacokinetic studies were conducted in 17 asymptomatic hemophilic patients, comparing pharmacokinetics of plasma-derived Antihemophilic Factor (Human) (pdAHF) and HELIXATE.[2] The mean biologic half-life of rAHF was 15.8 hours. The mean biologic half-life of pdAHF in the same individuals was 13.9 hours. A similar degree of shortening of the activated partial thromboplastin time was seen with both rAHF and pdAHF. The mean in vivo recovery of rAHF was similar to pdAHF, with a linear dose-response relationship. The recovery and half-life of rAHF was consistent with initial results following 13 weeks of exclusive treatment with HELIXATE. Subsequently, 826 recovery studies were conducted in 58 hemophilic patients participating in later clinical studies. Mean recovery from this group was 2.48% per IU/kg infused.

Fourteen (14) subjects from initial pharmacokinetic studies commenced home treatment with rAHF. Forty-four (44) additional subjects were then enrolled who treated themselves at home exclusively with rAHF. A total of 12,730 infusions have been administered under this portion of the study, of which 1,021 were given in clinic for recovery studies, 7,339 were given for treatment of bleeds, 4,361 were given as prophylaxis, 5 for minor surgery not requiring hospitalization, and 4 for unspecified reason.

Forty-eight (48) patients have received rAHF on 63 occasions for surgical procedures or in-hospital treatment of serious hemorrhage. Eleven (11) received rAHF for the first time in this study, while 37 were already on study or study participants under an investigation of previously untreated patients. Hemostasis has been satisfactory in all cases, with no adverse reactions.

In a study of previously untreated patients, a total of 3,254 infusions have been administered to 96 patients over a 48-month enrollment period. Hemostasis was successfully achieved in all cases.

During the analytical characterization of Antihemophilic Factor (Recombinant), HELIXATE®, analyses for carbohydrate structure revealed the presence of terminal galactose α1—3 galactose residues. Since naturally occurring antibody to this structure has been reported in humans, a trial in 18 patients was performed in which the half-life and recovery of rAHF with high levels of this carbohydrate residue was compared to that with HELIXATE, which contains low levels of this structure. As in the normal population, all patients had preexisting endogenous antibody to galactose α1—3 galactose in titers ranging from 1:320 to 1:5120 and no significant change in antibody level was noted during the study. While the mean recovery for HELIXATE in the group of 2.76%/IU/kg (N = 43), was significantly different from that of rAHF with high levels of residues, 2.43%/IU/kg (N = 18), p = 0.0001), the recovery for rAHF with high levels of galactose α1—3 galactose is not significantly different from the 2.48%/IU/kg recovery obtained in the larger study from the 58 patients treated with HELIXATE mentioned above. Based on these results, the galactose α1—3 galactose residue appears to have no clinical significance.

INDICATIONS AND USAGE

HELIXATE is indicated for the treatment of classical hemophilia (hemophilia A) in which there is a demonstrated deficiency of activity of the plasma clotting factor, factor VIII. HELIXATE provides a means of temporarily replacing the missing clotting factor in order to correct or prevent bleeding episodes, or in order to perform emergency and elective surgery in hemophiliacs.

HELIXATE can also be used for treatment of hemophilia A in certain patients with inhibitors to factor VIII. In clinical studies of HELIXATE, patients who developed inhibitors on study continued to manifest a clinical response when inhibitor titers were less than 10 Bethesda Units (B.U.) per mL. When an inhibitor is present, the dosage requirement for factor VIII is variable. The dosage can be determined only by clinical response, and by monitoring of circulating factor VIII levels after treatment (see **DOSAGE AND ADMINISTRATION**).

HELIXATE does not contain von Willebrand's factor and therefore is not indicated for the treatment of von Willebrand's disease.

CONTRAINDICATIONS

Due to the fact that Antihemophilic Factor (Recombinant) contains trace amounts of mouse protein (maximum 0.02 ng/IU rAHF) and hamster protein (maximum 0.04 ng/IU rAHF), HELIXATE should be administered with caution to individuals with previous hypersensitivity to pdAHF or known hypersensitivity to biologic preparations with trace amounts of murine or hamster proteins.

Assays to detect seroconversion to mouse and hamster protein were conducted on all patients on study. No patient has developed specific antibody titers against these proteins after commencing study, and no allergic reactions have been associated with rAHF infusions. Although no reactions were observed, patients should be warned of the theoretical possibility of a hypersensitivity reaction, and alerted to the early signs of such a reaction (e.g., hives, generalized urticaria, wheezing and hypotension). Patients should be advised to discontinue use of the product and contact their physician if such symptoms occur.

WARNINGS

None.

PRECAUTIONS

General

HELIXATE is intended for the treatment of bleeding disorders arising from a deficiency in factor VIII. This deficiency should be proven prior to administering HELIXATE.

The development of circulating neutralizing antibodies to factor VIII may occur during the treatment of patients with hemophilia A. In a study of previously untreated patients, inhibitor antibodies have developed in 17 of the 92 patients (18.5%) who have had at least one follow-up titer. The incidence of antibodies is 15/56 (26.7%) in patients with severe disease (<2% factor VIII), 2/18 (11%) in patients with moderate disease (2–5% factor VIII) and 0/18 in patients with mild disease (>5% factor VIII). Ten of the antibodies were high titer (>10 Bethesda Units), three were low titer, and four were low titer and transient. Studies most closely resembling the design of the study of inhibitor development with Antihemophilic Factor (Recombinant), HELIXATE® have reported incidences of inhibitor formation ranging between 19.4 and 52% for patients treated with pdAHF. The incidence of inhibitor formation in previously untreated patients treated with HELIXATE appears to be consistent with that reported in the literature, however the true immunogenicity of HELIXATE is not known at present. Patients treated with rAHF should be carefully monitored for the development of antibodies to rAHF by appropriate clinical observation and laboratory tests.

Product administration and handling of the infusion set and needles must be done with caution. Percutaneous puncture with a needle contaminated with blood can transmit infectious virus including HIV (AIDS) and hepatitis. Obtain immediate medical attention if injury occurs.

Place needles in sharps container after single use. Discard all equipment including any reconstituted HELIXATE product in accordance with biohazard procedures.

Carcinogenesis, Mutagenesis, Impairment of Fertility

In vitro evaluation of the mutagenic potential of HELIXATE failed to demonstrate reverse mutation or chromosomal aberrations at doses substantially greater than the maximum expected clinical dose. In vivo evaluation of rAHF using doses ranging between 10 and 40 times the expected clinical maximum also indicated that HELIXATE does not possess a mutagenic potential. Long-term investigations of carcinogenic potential in animals have not been performed.

Pediatric Use

HELIXATE has been proven to be safe and efficacious in newborns and children while under investigation as previously treated (n=21) and previously untreated patients (n=96) (see CLINICAL PHARMACOLOGY and PRECAUTIONS).

Pregnancy Category C

Animal reproduction studies have not been conducted with HELIXATE. It is also not known whether HELIXATE can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. HELIXATE should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

During the clinical studies conducted in previously treated patients, 47 out of 12,932 infusions (0.36%) were associated with 58 reported minor adverse reactions. Of these, 19 reactions were local to the injection site (e.g., burning, pruritus, erythema); and 39 were systemic complaints (dizziness, nausea, chest discomfort, sore throat, cold feet, unusual taste in mouth, and slight decrease in blood pressure). In the study with previously untreated patients, 3,254 infusions have been associated with 11 minor adverse reactions (0.34%): two reports of erythema at the injection site, one of facial flushing related to the infusion, one report of diarrhea, two reports of nonspecific rash, two reports of fever, and three reports of emesis. No serious reactions have been reported, and all reactions have been self-limited.

DOSAGE AND ADMINISTRATION

Each bottle of HELIXATE has the rAHF content in international units per bottle stated on the label of the bottle. The reconstituted product must be administered intravenously by either direct syringe or drip infusion. The product must be administered within 3 hours after reconstitution.

General Approach to Treatment and Assessment of Treatment Efficacy

The dosages described below are presented as general guidance. It should be emphasized that the dosage of HELIXATE required for hemostasis must be individualized according to the needs of the patient, the severity of the deficiency, the severity of the hemorrhage, the presence of inhibitors, and the factor VIII level desired. It is often critical to follow the course of therapy with factor VIII level assays. The clinical effect of HELIXATE is the most important element in evaluating the effectiveness of treatment. It may be necessary to administer more HELIXATE than would be estimated in order to attain satisfactory clinical results. If the calculated dose fails to attain the expected factor VIII levels, or if bleeding is not controlled after administration of the calculated dosage, the presence of a circulating inhibitor in the patient should be suspected. Its presence should be substantiated and the inhibitor level quantitated by appropriate laboratory tests. When an inhibitor is present, the dosage requirement for rAHF is extremely variable and the dosage can be determined only by the clinical response.

Some patients with low titer inhibitors (<10 B.U.) can be successfully treated with factor VIII without a resultant anamnestic rise in inhibitor titer.[7] Factor VIII levels and clinical response to treatment must be assessed to insure adequate response. Use of alternative treatment products, such as Factor IX Complex concentrates, Antihemophilic Factor (Porcine) or Anti-Inhibitor Coagulant Complex, may be necessary for patients with anamnestic responses to factor VIII treatment and/or high titer inhibitors.

Calculation of Dosage

The in vivo percent elevation in factor VIII level can be estimated by multiplying the dose of rAHF per kilogram of body weight (IU/kg) by 2%. This method of calculation is based on clinical findings by Abildgaard et al.[8] and is illustrated in the following examples:

[See first table above]

[See second table above]

or

[See third table above]

[See fourth table above]

The dosage necessary to achieve hemostasis depends upon the type and severity of the bleeding episode, according to the following general guidelines:

Mild Hemorrhage

Mild superficial or early hemorrhages may respond to a single dose of 10 IU per kg,[9] leading to an in vivo rise of approximately 20% in the factor VIII level. Therapy need not be repeated unless there is evidence of further bleeding.

Moderate Hemorrhage

For more serious bleeding episodes (e.g., definite hemarthroses, known trauma), the factor VIII level should be raised to 30–50% by administering approximately 15–25 IU per kg. If further therapy is required, a repeat infusion can be given at 12–24 hours.[10]

Severe Hemorrhage

In patients with life-threatening bleeding or possible hemorrhage involving vital structures (e.g., central nervous system, retropharyngeal and retroperitoneal spaces, iliopsoas sheath), the factor VIII level should be raised to 80–100% of normal in order to achieve hemostasis. This may be achieved with an initial rAHF (Antihemophilic Factor (Recombinant), HELIXATE®) dose of 40–50 IU per kg and a maintenance dose of 20–25 IU per kg every 8–12 hours.[11, 12]

Surgery

For major surgical procedures, the factor VIII level should be raised to approximately 100% by giving a preoperative dose of 50 IU/kg. The factor VIII level should be checked to assure that the expected level is achieved before the patient goes to surgery. In order to maintain hemostatic levels, repeat infusions may be necessary every 6 to 12 hours initially, and for a total of 10 to 14 days until healing is complete. The intensity of factor VIII replacement therapy required depends on the type of surgery and postoperative regimen employed. For minor surgical procedures, less intensive treatment schedules may provide adequate hemostasis.[11, 12]

Prophylaxis

Factor VIII concentrates may also be administered on a regular schedule for prophylaxis of bleeding, as reported by Nilsson et al.[13]

Reconstitution

Vacuum Transfer

1. Warm the unopened diluent and the concentrate to room temperature (NMT 37°C, 99°F).
2. After removing the plastic flip-top caps (Fig. A), aseptically cleanse the rubber stoppers of both bottles.
3. Remove the protective cover from the plastic transfer needle cartridge with tamper-proof seal and penetrate the stopper of the diluent bottle (Fig. B).
4. Remove the remaining portion of the plastic cartridge, invert the diluent bottle and penetrate the rubber seal on the concentrate bottle (Fig. C) with the needle at an angle.

   Alternate method of transferring sterile water: With a sterile needle and syringe, withdraw the appropriate volume of diluent and transfer to the bottle of lyophilized concentrate.
5. The vacuum will draw the diluent into the concentrate bottle. Hold the diluent bottle at an angle to the concentrate bottle in order to direct the jet of diluent against the wall of the concentrate bottle (Fig. C). Avoid excessive foaming.
6. After removing the diluent bottle and transfer needle (Fig. D), swirl continuously until completely dissolved (Fig. E).
7. After the concentrate powder is completely dissolved, withdraw solution into the syringe through the filter needle which is supplied in the package (Fig. F). Replace the filter needle with the administration set provided and inject intravenously.
8. If the same patient is to receive more than one bottle, the contents of two bottles may be drawn into the same syringe through a separate unused filter needle before attaching the vein needle.

[See figures A-F in next column]

Rate of Administration

The rate of administration should be adapted to the response of the individual patient, but administration of the entire dose in 5 to 10 minutes or less is well tolerated. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

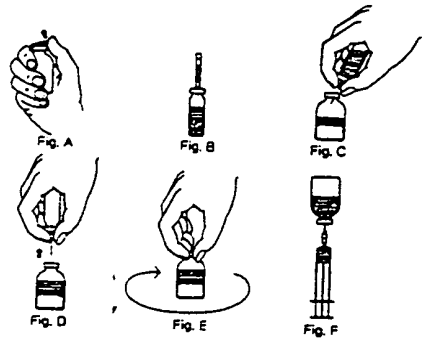
Expected % factor VIII increase = $\frac{\text{\# units administered} \times 2\%/\text{IU/kg}}{\text{body weight (kg)}}$

Example for a 70 kg adult: = $\frac{1400 \text{ IU} \times 2\%/\text{IU/kg}}{70 \text{ kg}}$ = 40%

Dosage required (IU) = $\frac{\text{body weight (kg)} \times \text{desired \% factor VIII increase}}{2\%/\text{IU/kg}}$

Example for a 15 kg child: = $\frac{15 \text{ kg} \times 100\%}{2\%/\text{IU/kg}}$ = 750 IU required

Product Code	Approximate Factor VIII Activity	Diluent
NDC 0053-8120-01	250 IU	2.5 mL
NDC 0053-8120-02	500 IU	5 mL
NDC 0053-8120-04	1000 IU	10 mL



HOW SUPPLIED

Antihemophilic Factor (Recombinant), HELIXATE® is supplied in the following single use bottles with the total units of factor VIII activity stated on the label of each bottle. A suitable volume of Sterile Water for Injection, USP, a sterile double-ended transfer needle, a sterile filter needle, and a sterile administration set are provided.

[See fifth table above]

STORAGE

HELIXATE should be stored under refrigeration (2°–8°C; 36°–46°F). Storage of lyophilized powder at room temperature (up to 25°C or 77°F) for 3 months, such as in home treatment situations, may be done without loss of factor VIII activity. Freezing should be avoided, as breakage of the diluent bottle might occur. Do not use beyond the expiration date indicated on the bottle.

CAUTION

U.S. federal law prohibits dispensing without prescription.

LIMITED WARRANTY

A number of factors beyond our control could reduce the efficacy of this product or even result in an ill effect following its use. These include improper storage and handling of the product after it leaves our hands, diagnosis, dosage, method of administration, and biological differences in individual patients. Because of these factors, it is important that this product be stored properly, and that the directions be followed carefully during use.

No warranty, express or implied, including any warranty of merchantability or fitness is made. Representatives of the Company are not authorized to vary the terms or the contents of the printed labeling, including the package insert for this product, except by printed notice from the Company's headquarters. The prescriber and use of this product must accept the terms hereof.

REFERENCES

1. Lawn RM, Vehar GA: The molecular genetics of hemophilia. *Sci Am* 254(3):48–54, 1986.
2. Schwartz RS, Abildgaard CF, Aledort LM, et al: Human recombinant DNA-derived antihemophilic factor (factor VIII) in the treatment of hemophilia A. *N Engl J Med* 323(26):1800–5, 1990.
3. Lusher JM: Viral safety and inhibitor development associated with monoclonal antibody-purified FVIIIc. *Ann Hematol* 63(3):138–41, 1991.
4. Addiego JE Jr, Gomperts E, Liu S-L, et al: Treatment of hemophilia A with a highly purified factor VIII concentrate prepared by anti-FVIIIc immunoaffinity chromatography. *Thromb Haemost* 67(1):19–27, 1992.

Continued on next page

EXHIBIT 3

United States Patent [19]

Zimmerman et al.

[11] 4,361,509

[45] Nov. 30, 1982

[54] ULTRAPURIFICATION OF FACTOR VIII USING MONOCLONAL ANTIBODIES

[75] Inventors: Theodore S. Zimmerman; Carol A. Fulcher, both of La Jolla, Calif.

[73] Assignee: Scripps Clinic and Research Foundation, La Jolla, Calif.

[21] Appl. No.: 330,105

[22] Filed: Dec. 14, 1981

[51] Int. Cl.³ C07G 7/00

[52] U.S. Cl. ..... 260/112 B; 424/101; 424/85

[58] Field of Search ..... 260/112 B; 424/101, 424/85

[56] References Cited

U.S. PATENT DOCUMENTS

3,631,018	12/1971	Shanbrom et al.	260/112 B
3,652,530	3/1972	Johnson et al.	260/112 B
3,682,881	8/1972	Fekete et al.	260/112 B
4,069,216	1/1978	Shanbrom	260/112 B
4,137,223	1/1979	Shanbrom et al.	260/112 B
4,188,318	2/1980	Shanbrom	260/112 B
4,203,891	5/1980	Rock	260/112 B

OTHER PUBLICATIONS

J. of Lab. Clin. Med., vol. 93, p. 40, (1979), Tuddenham et al.

Primary Examiner—Howard E. Schain

[57] ABSTRACT

A method of preparing high purity procoagulant protein comprising the steps of (a) adsorbing a VIII:C-/VIII:RP complex from a plasma or commercial concentrate source of factor VIII onto agarose beads bound to a monoclonal antibody specific to VIII:RP, (b) eluting VIII:C with a salt solution, (c) adsorbing the eluted VIII:C on an animohexyl agarose column and eluting the VIII:C with a salt solution.

16 Claims, No Drawings

ULTRAPURIFICATION OF FACTOR VIII USING MONOCLONAL ANTIBODIES

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to a method of separating and purifying factor VIII procoagulant activity protein. More specifically, high purity factor VIII procoagulant activity protein is separated from von Willebrand Factor by a two step chromatographic adsorption and concentration technique from plasma or concentrate.

2. Description of the Prior Art

The isolation of the antihemophilic factor from blood plasma has been described in the literature. The precise structure of the antihemophilic factor, also referred to as factor VIII procoagulant activity protein (factor VIII), has not yet been identified, due in part to the unavailability of sufficient quantities of pure material with which to conduct further studies. The limited availability of pure material and its existence in a dilute state has also hindered its use in therapeutic applications.

Factor VIII procoagulant activity protein functions to correct the clotting defect in hemophilic plasma. It circulates in plasma complexed with the von Willebrand factor protein. The latter can alter the platelet function defect in von Willebrand's disease. That portion of the factor VIII von Willebrand factor complex having coagulant activity is referred to as factor VIII procoagulant activity protein, factor VIII-clotting activity or simply VIII:C (the designation of "VIII:C" will be used hereinafter to identify the portion of the factor VIII molecule with such clotting activity.) The other portion of the factor VIII von Willebrand factor complex having the ability to correct the platelet function defect in von Willebrand's disease is referred to as von Willebrand factor, factor VIII-related antigen, VIIIR:Ag, VIII:RP factor. (The description "VIII:RP" will be used hereinafter to identify the platelet correction function of the factor VIII molecule). Although yet unproven, there is evidence to support the conclusion that VIII:C exhibits properties and the behavior of a small molecule which is combined with VIII:RP as a non-covalent complex. There is also a basis for the contention that the properties associated with both VIII:C and VIII:RP may also be a single molecule which under appropriate conditions may be cleaved, yielding two fragments.

In view of the need for identifying the structures of the factor VIII/von Willebrand factor complex, VIII:C and VIII:RP and the important pharmaceutical value of the coagulant activity ascribable to VIII:C, numerous attempts have been made to purify factor VIII and to separate and concentrate VIII:C and VIII:RP. The techniques used are based generally on either immunoadsorption or ion exchange chromatography. Such techniques as heretofore used have had limited success due to the difficulty of desorbing the proteins from the charged ionic material in an undamaged condition or recovering same in suitable quantities.

One such method for separating VIII:C from VIII:RP utilizing immunoadsorbent chromatography has been reported by E. G. D. Tuddenham et al, "The Properties of Factor VIII Coagulant Activity Prepared by Immunoadsorbent Chromatography", JOURNAL OF LABORATORY CLINICAL MEDICINE, Vol. 93, p. 40 (1979). The reported method is a one-step separation of VIII:C from nearly all VIII:RP and from most other plasma proteins employing a chromatographic column packed with agarose beads to which polyclonal antisera to VIII:RP (anti-VIII:RP) are coupled. Factor VIII/von Willebrand factor containing plasma is passed through the column which adsorbs both VIII:C and VIII:RP. Other unwanted plasma proteins are removed from the column by washing with buffered saline solution and the desired VIII:C is obtained by subsequent elution with a calcium-ion gradient. Although it is stated to be an improvement in both purity and yield of VIII:C, when compared to the previously known methods, it is also stated that the resulting product also contains VIII:RP and other plasma proteins. Such contaminants may be attributable to the use of polyclonal antisera bound to the agarose beads. Since a majority of the immunoglobulins from which the antisera are constituted are not specific to VIII:RP, the effective number of sites where antibodies specific to VIII:RP may be bound to agarose is relatively small due to competition between the antisera for a finite number of bonding sites on the agarose.

Another method for separating VIII:C from VIII:RP and ristocetin co-factor by a chromatographic technique employing aminohexyl-substituted agarose has been described by D. E. G. Austen, "The Chromatographic Separation of Factor VIII on Aminohexyl Sepharose," BRITISH JOURNAL OF HAEMATOLOGY, Vol. 43, p. 669 (1979). The described method is stated to be an improved method for the component parts of both human and porcine factor VIII/von Willebrand factor. This method, however, also suffers from the fact that contaminants are present in the resulting product. In both the Tuddenham et al and Austen methods a contaminated product, which is more dilute than is normally desired, is formed.

Hence, it is clear that there still exists a need for an improved method for separating and purifying VIII:C from VIII:RP using plasma or concentrates. Therefore, it is an object of the present invention to satisfy such a need.

SUMMARY OF THE INVENTION

The present invention relates to a method of separation of the component molecules of the factor VIII/von Willebrand factor complex, VIII:C and VIII:RP, and the purification and concentration of the pro-coagulant activity protein VIII:C. The method achieves the object of producing highly purified VIII:C using a two step procedure.

The first step involves immunoadsorption of factor VIII from plasma or a commercial concentrate. The adsorbent employed comprises a monoclonal antibody specific to VIII:RP which is bound to a suitable substrate such as, agarose beads. After the VIII:C/VIII:RP is initially adsorbed, the substrate particles are washed extensively with a buffer solution to remove unadsorbed protein. The adsorbed material is then treated with a calcium ion containing solution to elute the adsorbed VIII:C. The VIII:RP portion remains adsorbed on the anti-VIII:RP bound material. At this point about 40–60% of the VIII:C initially adsorbed is recovered in a highly purified state. However, the procoagulant activity protein recovered, although extremely pure, i.e., largely free from contaminants, is too dilute to be of significant therapeutic value.

The second step of the present process is directed to substantially concentrating the recovered purified VIII:C using a technique which may be characterized as affinity chromatography.

The VIII:C solution obtained from the first step of the present process having a potency of approximately 10–20 International Units (hereinafter "units") is processed in a column containing aminohexyl substituted agarose. The column is then washed with a buffer solution and the VIII:C is eluted with a calcium ion-containing solution to yield a VIII:C concentration in excess of 1000 units per ml, and being greater than 160,000 fold purified from plasma. Thus, the present method yields unexpectedly high purity procoagulant activity protein in a highly concentrated and therapeutically useful state. Methods used heretofore fail to achieve such notable results for several reasons. The method of Tuddenham et al, described earlier, employs bound polyclonal antisera instead of the specific and highly selective monoclonal antibodies to VIII:RP as used in the present invention. As a result, fewer specific antibodies to VIII:RP are coupled for a given weight of agarose. In the method of the present invention monoclonal antibodies are exclusively bound to a relatively inert substrate. When the method of Tuddenham et al is used only 2.6 to 6.4 units of VIII:RP per ml of immunoglobulin-agarose beads (equivalent to 53.1–82.9% of the amount applied to the column) are removed. This compares to greater than 1000 units per ml of beads (or 90–100% of the VIII:RP which is applied to the column) which is recovered when the monoclonal antibody immunoadsorbent of the present invention is employed. This ability to adsorb more VIII:C/VIII:RP (factor VIII/von Willebrand factor) per ml of beads accordingly results in a higher concentration of VIII:C when it is subsequently eluted from the immunoadsorbent. Thus, 10–20 units of VIII:C per ml of eluant are obtained with the present invention, in contract to 0.5–1.25 units per ml of eluant with the Tuddenham et al method.

The present method also permits the selection of a monoclonal antibody having a high affinity for VIII:RP; however, the use of polyclonal antibodies results in varying affinities. It should be realized that there is an indirect relationship between the affinity of the bound antibody for VIII:RP and the elution of VIII:RP. Thus, the higher the affinity of the antibody for VIII:RP, the less VIII:RP will be present with VIII:C in the eluant. The present invention also makes it possible to produce an unlimited supply of the specified monoclonal antibody, thus eliminating variations among different batches.

Although Austen, as earlier described, has reported the use of aminohexyl-agarose to separate VIII:C from VIII:RP, such a material has not heretofore been used to concentrate VIII:C following a separation and purification step. Heretofore, the highest VIII:C concentrations achieved by using aminohexyl agarose in chromatography were 0.53 units per ml of eluant for human protein and 2.38 per ml of eluant for porcine VIII:C. The present method permits concentrations several orders of magnitude greater than these. Perhaps of even greater significance, is the fact that the present invention provides for a greater purification of human VIII:C than has ever been reported (164,000 vs 17,000 fold over plasma). The present method, which is described in more detail hereinafter, yields VIII:C with a specific activity of 2,300 units/mg when commercial concentrate is used. This corresponds to a 164,000 fold purification from plasma. The ratio of VIII:C to VIII:RP is greater than 10^5 as compared to the ratio in plasma.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following description provides details of the manner in which the embodiments of the present invention may be made and used in order to achieve the separation, purification and concentration of VIII:C to a degree of purity and concentration not known heretofore. This description, while exemplary of the present invention, is not to be construed as specifically limiting the invention and such variations which would be within the purview of one skilled in this art are to be considered to fall within the scope of this invention.

A. Preparation of Monoclonal Antibody to VIII:RP

The monoclonal antibody to VIII:RP which is subsequently bound to the separation substrate may be prepared in a stepwise procedure starting with a highly purified preparation of factor VIII/von Willebrand factor (VIII:C/VIII:RP complex). The purification for immunization is accomplished with material obtained from a plasma source. Less highly purified material for coating polyvinyl plates is obtained in higher concentration from commercial extracts such as FACTOR-ATE (trademark of Armour Pharmaceutical Co., Tuckahoe, N.Y.) or Hemophil (trademark of Hyland Laboratories, Costa Mesa, California). Purification is performed by a standard agarose-gel filtration of cryoprecipitate, such as that described by Zimmerman and Roberts, "Factor VIII Related Antigen", appearing in IMMUNOASSAYS: CLINICAL LABORATORY TECHNIQUES FOR THE 1980's, R. M. Nakamura et al, eds., Alan R. Liss, Inc., New York, pp. 339–349 (1980). Mice were injected with highly purified factor VIII/von Willebrand factor obtained from plasma according to the following procedure. On day zero, the mice are injected intraperitoneally with a composition prepared by dissolving (or suspending) 10 Mg of the protein in 0.1 ml of buffer containing 0.05 M Tris, 0.15 M sodium chioride, 0.02% sodium azide, 1 mM phenyl methyl sulfonyl fluoride, trasylol 10 units/ml at pH7.3. and shaking with an equal volume of complete Freund's adjuvant. On day 14, the mice are again injected with the same material except that incomplete Freund's adjuvant is substituted for complete Freund's adjuvant. On day 21, the injection of day 14 is repeated. On day 38, the mice are injected with purified VIII:C/VIII:RP only. On day 42, the spleens of the mice are removed and fused according to a standard procedure, of the type described by J. P. Brown et al "Protein Antigens of Normal and Malignant Human Cells Identified by Immunoprecipitation with Monoclonal Antibodies", JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 225, pp. 4980–4983 (1980). The standard technique is varied only to the extent that 35% polyethylene glycol 1000 is substituted for 50% polyethylene glycol. A radioimmunoassay method for clones producing antibody to VIII:RP is performed according to the following procedure. Polyvinyl plates with a "V" bottom, flexible type are coated with 0.1 ml of factor VIII purified from commercial extract according to the procedure indicated above and having a concentration of 0.125 mg/ml of protein. The plates are blocked with albumin, washed with buffer and incubated with the culture fluids from the clones to be tested. The plates

are then washed and reacted with rabbit anti-mouse IgG antiserum, washed a second time and ^{125}I labeled goat anti-rabbit IgG antiserum is added to the wells and incubated. The plates are again washed, then dried and the wells cut-out and counted. After determining the clones which are positive they are subcloned at least twice and stable clones producing antibody to VIII:RP are then injected into the peritoneal cavities of Balb/C mice which have been pretreated intraperitoneally with 0.5 ml of pristane at least four days prior to injection of cells. Hybridoma cells are injected at concentrations of approximately 5×10^6 cells per mouse in 0.5 ml of Delbecco's modified Eagle's medium without fetal bovine serum. The mice are tapped when bloated and ascites fluid is collected in heparin at approximately 10 units/ml. Ascites fluid from multiple mice is pooled to provide a convenient volume for subsequent isolation of the monoclonal IgG. If the heparinized ascites fluid is not used immediately, it may be stored at −70° C. and thawed just prior to use. The final yield of IgG from the ascites fluid is approximately 1 g of IgG per 100 ml of ascites fluid.

The specificity of the monoclonal IgG for the purpose of purifying VIII:C may be assessed by coupling the IgG to a separation substrate medium, in the manner described hereinafter, and demonstrating that the bound IgG removes both VIII:RP and VIII:C from plasma and that the VIII:C may be subsequently eluted with a solution containing calcium ions while the VIII:RP remains complexed to the monoclonal IgG which is bound to the solid-state substrate.

The monoclonal IgG, which is to be used subsequently to prepare the immunoadsorbent, may be isolated from heparinized pooled ascites fluid immediately after collection or a frozen portion of the stored solution may be thawed. Regardless of whether fresh or frozen material is used, the solution is brought to 4° C. and treated with an equal volume of phosphate buffered saline solution (PBS), the composition of which is set forth below. The diluted ascites is precipitated by dropwise addition with stirring at 4° C. of an equal volume of saturated ammonium sulfate (SAS); prepared by boiling an excess of ammonium sulfate in water, cooling to 4° C., filtering undissolved crystals and adjusting the pH to 7.0 with ammonium hydroxide. The precipitate and its supernatant liquid are stirred for at least 2 hours and centrifuged at 4° C. Centrifugations are preferably carried out at 14,000 rpm for 60 minutes ($30,000\times g$). The supernatant solution of ascites is precipitated twice more with SAS and the mixture of precipitate and supernatant liquid stirred and centrifuged in the same manner as in the first cycle. The pellets resulting from the third precipitation are resuspended in a volume of PBS equal to that of the diluted ascites fluid and then disalyzed exhaustively against PBS. Clots appearing in the dialysis bags are removed by centrifugation at 20° C. The dialyzed IgG is adsorbed by stirring it with a 5% aqueous solution of aluminum hydroxide at room temperature and centrifuging at 20° C. after adsorption. The adsorption treatment is repeated at least three more times using 2.5% aluminum hydroxide solution for each treatment after the first. The adsorbed IgG is brought to 4° C. and reprecipitated once with SAS as described above. The precipitated pellets may be stored at −20° C. until used.

B. Preparation of the Immunoadsorbent

The immunoadsorbent is prepared by suitably preparing the monoclonal IgG for coupling, preparing the solid substrate for coupling and reacting the two components to bind the former to the later.

(i) Preparation of IgG for Coupling

Either freshly precipitated IgG may be used or previously frozen precipitate may be thawed for use. The material is then dialyzed against PBS, and while still in the PBS, the volume and IgG concentration ($A_{280}/1.4$=mg./ml IgG) are determined. The IgG is then treated with between 10 and 30 microliters, preferably 20 microliters, of diisopropylfluorophosphate per 50 ml of IgG solution. The resulting solution is stirred at room temperature in a hood for 30 minutes and the treated IgG, immediately prior to use, is dialyzed overnight against coupling buffer. The coupling buffer found most suitable is a 0.25 M sodium bicarbonate solution adjusted to a pH of 9, preferably with sodium hydroxide.

(ii) Preparation of Solid Substrate for Coupling

Although the monoclonal antibody may be bound to any material which does not have a high affinity for protein, particularly factor VIII itself, such materials as glass beads, agarose and derivatives thereof are preferred. Most preferred is a crosslinked agarose available commercially as a gel known as Sepharose CL2B (trademark of Pharmacia Fine Chemicals, Piscataway, N.J.).

The method of preparing the preferred immunoadsorbent resin is generally the same as that disclosed in the literature, such as the method of J. Porath et al, JOURNAL OF CHROMATOGRAPHY, Vol. 86, pp. 53–56 (1973). The method found most suitable is as follows: a volume of about 2 liters of Sepharose CL2B is placed in an acid-cleaned 2 liter sintered glass filter funnel. The resin is washed with water and filtered to a moist cake. The washed resin is placed in a large (approximately 4 liter) glass beaker equipped with a magnetic stirring bar. To the resin is then added 750 ml of cold potassium phosphate buffer solution, prepared by mixing one part of a 5 M dibasic potassium phosphate solution with two parts of 5 M tribasic potassium phosphate solution. Sufficient cold water is added to bring the final volume to 3 liters. The mixture is then chilled to 4° C. and maintained at between 4°–10° C. in an ice-water bath placed on a magnetic stirring plate. In a hood, cyanogen bromide is added to 300 ml of water in a stoppered glass bottle containing a magnetic stirring bar. The mixture is rapidly stirred until solution results. The cyanogen bromide solution is then added with stirring over a 2 minute period to the cold Sepharose mixture. Stirring is continued for an additional 8 minutes and then transferred to a chilled 2 liter sintered glass filter funnel supported in a 4 liter vacuum flask. The cyanogen bromide treated resin is then washed with approximately 20 liters of cold water or until the pH of the filtrate is neutral. The washed resin is then quickly equilibrated with cold coupling buffer and then transferred to a 4 liter plastic beaker equipped with a large magnetic stirring bar.

(iii) Coupling the Monoclonal Antibody to the Solid Substrate

The solid substrate resin, prepared as indicated above, is ready to be used when it is equibrated with coupling buffer and should not be stored thereafter. Accordingly, the resin mixture is combined with the

IgG which was previously dialyzed overnight against coupling buffer. The combined resin/IgG suspended mixture is stirred at 4° C. for a period of about 24 hours. The A_{280} of an undiluted sample of the supernatant coupling liquid may be determined using bovine serum albumin (BSA) as a standard or Bio-Rad protein assay (Bradford reagent) with BSA as standard. The percentage ligand which is coupled may then be calculated. When the above described procedure is followed, this is usually about 95%. Any remaining active sites on the resin not coupled to antibody may be blocked by washing the resin on a sintered glass filter funnel with cold coupling buffer containing 0.1 M glycine. The resin is then resuspended in this solution to a final volume equal to that when the resin and antibody, each in coupling buffer, were combined. The suspension is stirred slowly overnight at 4° C. The resin is then washed thoroughly with VIII:C-buffer, the composition of which is given below. The coupled, blocked resin is then pre-eluted with VIII:C-buffer additionally containing 0.5 M calcium ions, preferably calcium chloride. The resin is again washed with VIII:C buffer alone and stored at 4° C. or in a continuously pumped column at room temperature until ready for use. The coupling density of IgG to SEPHAROSE should be 2–5 g, preferably 3–4 g IgG/liter of SEPHAROSE.

C. Separation and Purification of VIII:C

(i) Sample preparation of factor VIII, such as human and animal plasmas and commercial concentrates of factor VIII, may be employed in the present invention and the method is not limited as to a particular type of material. Preferred materials, and those which have demonstrated successful results, are porcine and human plasmas and commercially available concentrates of human factor VIII, such as FACTORATE available from Armour Pharmaceutical Co. The following description provides details for using both porcine plasma or commercial human concentrate such as FACTORATE:

FACTORATE is reconstituted by adding 25 ml portions of VIII:C-buffer to the contents of each of 20 bottles containing 400–500 VIIIC units per bottle (25 ml per bottle). The mixture is adjusted to a final volume of 1 liter with VIII:C-buffer. A sample aliquot of 0.5 ml may be removed for assay and the remaining material applied to the immunoadsorbent column overnight at a rate of approximately 60 ml/hour.

Porcine plasma, when not freshly drawn, is citrated by conventional means and stored frozen. When ready to be used it is thawed at a temperature of between 35°–40° C., preferably 37° C. and applied directly to the column at 60 ml/hour.

It should be noted that while the description of the present invention refers, and is directed primarily, to the use of immunoadsorbent coupled particles in a chromatography column, it is within the scope of this invention to perform batchwise separations by placing the antibody-bound resin particles in a suitable container and after adding reconstituted concentrate or plasma, VIII:C as outlined above and described in more detail below.

When the process is carried out in a chromatography process, the following embodiments are preferred:

The resin is placed in a column, such as an Amicon 86001, (trademark of Amicon Corp., Lexington, Mass.), equipped with a peristaltic pump and a high flow head. When concentrate is used as the source of factor VIII, for 20 bottles of diluted concentrate, approximately 1.5 liters of resin, prepared as indicated above, is used. When porcine plasma is used, 150 ml of resin is used for each liter of plasma.

After the sample is applied to the column, it is washed with 1 liter of VIII:C-buffer, followed by a second washing with VIII:C-buffer which additionally contains 0.5 M NaCl. Approximately 20 liters of saline-buffer is used when factor VIII is applied as concentrate and 20 bed volumes when porcine plasma is employed. Optimum results are obtained with a flow rate of 1 liter/hour.

Elution of purified VIII:C is accomplished with VIII:C-buffer containing calcium ions. Although a linear gradient, as taught by Tuddenham et al, supra, works well, it is not required in order to accomplish the object of this invention; a solution having a fixed calcium ion concentration is quite adequate. Thus, when VIII:C derived from concentrate is being eluted, VIII:C-buffer, 0.25 to 0.5 M with respect to calcium chloride, preferably 0.35 M, is used advantageously as a flow rate of between 450 to 750 ml/hour and preferably 600 ml/hour. When the VIII:C is obtained from porcine plasma, elution is performed with VIII:C-buffer being a calcium chloride concentration of between 0.35 and 0.7 M, preferably 0.5 M and at a flow rate of between 10 and 30 ml/hour, preferably 20 ml/hour. Fractions of 12 ml and 3 ml are collected for VIII:C originating from concentrate and porcine plasma, respectively. Those fractions containing at least 1.0 unit/ml of VIII:C activity are pooled and the total volume and activity of the pool determined.

The VIII:C pool is initially concentrated to 10–20 ml by a standard procedure such as pressure ultrafiltration. For this purpose, Amicon stirred cell in which a YM-10 membrane under 50 psi of nitrogen pressure has been found to work well. Slow stirring is continued for 30 minutes after nitrogen pressure is released, and the volume and activity of the concentrated pool are determined. The pool may be stored for a brief period, that is, overnight for example, if a temperature of 4° C. is maintained.

It may be noted that the immunoadsorbent column described above may be regenerated by treatment of the column with 2 bed volumes of 3 M aqueous sodium thiocyanate solution run at a flow rate of about 0.5–1 liter/hour to elute VIII:RP.

D. Concentration of Purified VIII:C

Although the VIII:C recovered from the separation from VIII:RP by means of the immunoadsorbent column is highly purified, it is still too dilute to be therapeutically useful. Further concentration and a four fold increase in purification when porcine plasma is used is accomplished by use of an aminohexyl agarose column which is prepared and used in the following manner:

(i) Preparation and/or Conditioning of a Aminohexyl Agarose Column:

Aminohexyl agarose is agarose which has been reacted with 1,6-diaminohexane to yield an agarose resin having a number of 6 carbon atom chains, each of which has a terminal amino group. It may be prepared according to the method described by Austen, supra, or acquired from a commercial supplier. One such material which has been used successfully in the present invention is available under the name of AH-SEPHAROSE 4B (trademark of Pharmacia Fine Chemicals, Piscataway, N.J.).

Whether prepared or purchased, the resin should be conditioned prior to use. This may be accomplished as follows, the volumes, amounts and dimensions being adjusted in proportion to the amount of material to be concentrated:

Approximately 1 gram of aminohexyl agarose (AH-SEPHAROSE 4B) is placed in a sintered glass filter funnel and washed with at least 200 ml of 0.5 M sodium chloride, while stirring. The resin is then equilibrated with VIII:C-buffer and packed in a column of approximately 0.9 cm diameter. A Bio-Rad Econo-Column with flow adapters has proven quite suitable for the type of use considered here. The bed volume of the packed column is approximately 4 ml.

(ii) Application to and Use of the Aminohexyl Agarose Column

The concentrated pool, prepared as described above, is diluted 1:10 in VIII:C-buffer to a final concentration of 100–200 ml when using the amounts of resin and column size as described in the immediately preceding section. The diluted pool is applied to the column at a flow rate of 200 ml/hour.

The column is then washed with VIII:C-buffer which contains calcium ions, preferably from calcium chloride. The solution should be between 0.01 M to 0.03 M, preferably 0.025 M with respect to calcium ions.

Elution of the concentrated VIII:C is achieved at a flow rate of between 5 to 20 ml/hour, preferably 10 ml/hour with VIII:C-buffer containing a higher concentration of calcium ions than was employed with the preceding washing step. Again, calcium chloride is the preferred source of calcium ions in a concentration of between 0.25 to 0.5 M, preferably 0.3 M. Fractions of 1 ml volume are collected and assayed as described below. Collected fractions may be stored at 4° C. or frozen. Preparations of VIII:C obtained from a porcine plasma source should be stabilized within 5 to 10% human serum albumin prior to storage.

Assays may be performed by diluting the fractions with VIII-C buffer if necessary and further diluting the fraction 1:100 in assay buffer prior to addition to the substrate. A standard partial thromboplastin time assay is employed.

The composition of the buffer solutions is as follows:

Phosphate Buffered Saline Solution:

1.6 g sodium phosphate, monobasic monohydrate
8.4 g sodium phosphate, dibasic anhydrous
61.4 sodium chloride
Water to 7 liters
pH of buffer is 7.2

VIII:C-Buffer

ml 0.02 M imidazole
ml 0.15 M sodium chloride
ml 0.10 M lysine
ml 0.02% sodium azide

pH of buffer is adjusted with concentrated hydrochloric acid to 6.8.

The data listed hereinafter in Tables I and II are representative of that obtained according to the present invention, as described above.

TABLE I

VIII:C Obtained From FACTORATE Concentrate as the Source of VIII:C/VIII:RP

	Volume (ml)	VIIIC (Units/ml)*	VIIIC (Total Units)	Protein (mg/ml)	Protein (Total mg)	Recovery (%)	Specific Activity (Units/mg)	From Plasma (Fold Purif.)
Sample Applied to Immunoadsorbent	500	18.8	9400	29	14,500	—	0.7	50
Pool resulting from Immunoadsorbent	1020	4.6	4692	—	—	50	—	—
Pool After Initial Concentration	20	134	2680	—	—	29 (57)	—	—
Sum Resulting from Aminohexyl Column	—	—	1576	—	—	17 (59)	—	—
Aminohexyl Fraction #3	0.95	1172	1112	0.51	0.48	12	2294	163,857
Aminohexyl Fraction #4	—	545	—	0.23	—	—	2370	169,285

*A frozen human plasma pool used as the standard for VIIIC assays and assigned the value of 1 human unit per ml.

TABLE II

VIII:C Obtained From Citrated Porcine Plasma

	Volume (ml)	VIIIC (Units/ml)	VIIIC (Total Units)	Protein (mg/ml)	Protein (Total mg)	Recovery (%)	Specific Activity (Units/mg)	From Plasma (Fold Purif.)
Sample Applied to Immunoadsorbent	1000	1*	1000	76	76,000	100	0.013	—
Pool Resulting from Immunoadsorbent	70	8.8	613	—	—	61	—	—
Pool After Initial Concentration	5.76	88	494.5	0.242	1.355	49.5	364	28,000
Sum Resulting from Aminohexyl Column	5.0	49	247	0.035	0.175	25	1413	109,000

*Porcine plasma used as the standard for VIIIC assays and assigned the value of 1 porcine VIIIC unit per ml.

Although only preferred embodiments are specifically illustrated and described herein, it will be appreciated that many modifications and variations of the present invention are possible in light of the above teachings and within the purview of the appended claims without departing from the spirit and intended scope of the invention.

What is claimed is:

1. An improved method of preparing Factor VIII procoagulant activity protein comprising the steps of

(a) adsorbing a VIII:C/VIII:RP complex from a plasma or commercial concentrate source onto particles bound to a monoclonal antibody specific to VIII:RP,

(b) eluting the VIII:C,

(c) adsorbing the VIII:C obtained in step (b) in another adsorption to concentrate and further purify same,

(d) eluting the adsorbed VIII:C, and

(e) recovering highly purified and concentrated VIII:C.

2. A method according to claim 1, wherein the elutant used in each of steps (b) and (d) is a saline solution.

3. The method according to claim 2, wherein the saline solution is calcium chloride.

4. The method according to claim 3, wherein the concentration of said calcium chloride solution used in steps (b) and (d) ranges from about 0.25 M to about 0.5 M.

5. The method according to claim 1, wherein said adsorbent particles in step (a) are agarose.

6. The method according to claim 1, wherein aminohexyl agarose is employed in step (c) as the adsorbent.

7. The method according to claim 6, wherein calcium chloride solution is employed as the elutant in steps (b) and (d), concentration of said calcium chloride solution ranging from about 0.25 M to about 0.5 M in step (b) and from about 0.25 M to about 0.5 M in step (d).

8. An improved immunoadsorbent for isolation and purification of VIII:C from VIII:C/VIII:RP comprising a monoclonal antibody specific to VIII:RP bound to solid particles.

9. The improved immunoadsorbent of claim 8, wherein said solid particles comprise a resin.

10. The improved immunoadsorbent of claim 9, wherein said resin comprises agarose.

11. The improved immunoadsorbent of claim 10, wherein said agarose is cross-linked agarose.

12. The improved immunoadsorbent of claim 11, wherein said immunoadsorbent has a coupling density of 3 to 4 g of monoclonal antibody per liter of agarose.

13. Highly purified and concentrated VIII:C prepared in accordance with the method of claim 1.

14. Highly purified and concentrated VIII:C prepared in accordance with the method of claim 6.

15. In a method for purifying Factor VIII procoagulant activity protein from plasma or concentrate, the improvement comprising the step of passing said plasma or concentrate through a chromatographic type column having adsorbent to which is bound monoclonal antibodies which is specific to VIII:RP and eluting the VIII-C therefrom.

16. The method according to claim 15, wherein said adsorbent is agarose and said elutant is a saline solution.

* * * * *

EXHIBIT 4

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	F.C. Prats
Art Unit	:	1651
Applicant	:	Bruce Joseph Roser
Serial No.	:	08/875,796
Filed	:	October 30, 1998
For	:	Dried Blood Factor Compositions Comprising Trehalose

DECLARATION OF ALAN P. MACKENZIE UNDER 37 CFR §1.132

I, hereby declare that:

I have the following educational background:

Univ. of London, England	B.Sc.	1951	General Science
Univ. of London, England	B.Sc.	1952	Chemistry
Univ. of London, England	Ph.D.	1957	Chemistry

Honors: Both B.Sc. degrees were taken with First Class Honours.

I have held the following Research and Professional Appointments:

Research Associate Professor, Center for Bioengineering, School of Medicine, University of Washington, Seattle, Washington.

Associate Professor, Center for Bioengineering, School of Medicine, University of Washington, Seattle, Washington.

Research Associate, High Voltage Electron Microscope Laboratory, University of Wisconsin, Madison, Wisconsin.

Associate Director, Cryobiology Research Institute, Madison, Wisconsin.

Associate Director of Research, American Foundation for Biological Research, Madison, Wisconsin.

Research Associate, American Foundation for Biological Research, Madison, Wisconsin.

Staff Scientist, Glaxo Research, Glaxo Laboratories, Ltd., Greenford, Middlesex, England.

The following are representative of my technical publications:

MacKenzie, A.P. and Luyet, B.J.: A collodion sandwich-film technique for the study of the growth of ice in very thin layers of aqueous solutions. In "Electron Microscopy, Vol. 2" (Proceedings of the Fifth International Congress for Electron Microscopy, Philadelphia, 1962). Ed.: S.S. Breese, Academic Press, New York, N.Y., p. P 2, 1962.

MacKenzie, A.P. and Luyet, B.J.: Electron microscope study of the structure of very rapidly frozen gelatin solutions. Biodynamica 9:47-69, 1962.

MacKenzie, A.P. and Luyet, B.J.: An electron microscope study of the fine structures of very rapidly frozen blood plasma. Biodynamica 9:147-164, 1963.

MacKenzie, A.P. and Luyet, B.J.: Apparatus for freeze-drying at very low, controlled temperatures. Biodynamica 9:177-191, 1964.

MacKenzie, A.P. and Luyet, B.J.: Apparatus for the automatic recording of freeze-drying rates at controlled specimen temperatures. Biodynamica 9:193-206, 1964.

MacKenzie, A.P. and Luyet, B.J.: Effect of recrystallization upon the velocity of freeze- drying. In "Progress in Refrigeration Science and Technology" (Proceedings of the XIth. International Congress of Refrigeration, Munich, 1963). Pergamon Press, New York, N.Y. VI C-2, pp. 1573-1581, 1965.

MacKenzie, A.P.: Factors affecting the mechanism of transformation of ice into water vapor in the freeze-drying process. Ann. N.Y. Acad. Sci. 125, Art. 2:522-547, 1965.

MacKenzie, A.P.: Basic principles of freeze-drying for pharmaceuticals. Bull. Parenteral Drug Assoc. 20:101-129, 1966.

MacKenzie, A.P. and Luyet, B.J.: Freeze-drying and protein denaturation in muscle tissue; losses in protein solubility. Nature 215:83-84, 1967.

MacKenzie, A.P. and Luyet, B.J.: Electron microscope study of recrystallization in rapidly frozen gelatin gels. Biodynamica, 10: 95-122, 1967.

MacKenzie, A.P. and Rapatz, G.L.. Transition from polyhedral to spheroidal ice crystals, and vice versa, in partly frozen aqueous systems. Biodynamica 10:219-227, 1968.

MacKenzie, A.P.: Death of frozen yeast in the course of slow warming. In "The Frozen Cell" (Proceedings of a CIBA Foundation Symposium). Ed.: G.E.W. Wolstenholme and M. O'Connor, J. and A. Churchill, London, pp. 89-96, 1970.

Arenberg, I.K., Marovitz, W.F. and MacKenzie, A.P.: Preparative techniques for the study of soft biological tissues in the scanning electron microscope: A comparison of air drying, low temperature evaporation and freeze-drying. In Proceedings of the Third Annual Stereoscan Colloquium." Kent Cambridge Scientific, Inc., Morton Grove, Ill., pp. 121-143, 1970.

George, R.P., Albrecht, R.M., Raper, K.B., Sachs, I.B. and MacKenzie, A.P.: Rapid freeze preparation of Dictyostelium discoideum for scanning electron nicroscopy. In Proceedings of the Third Annual Stereoscan Colloquium. Kent Cambridge Scientific, Inc., Morton Grove, Ill., pp. 159-165, 1970.

Rasmussen, D.H. and MacKenzie, A.P.: The glass transition in amorphous water. Application of the measurements to problems arising in cryobiology. J. Phys. Chem. 75:967-973, 1971.

Arenberg, I.K., Marovitz, W.F. and MacKenzie, A.P.: Preparative techniques for the study of soft biologic tissues in the scanning electron microscope. Trans. Amer. Acad. Ophthalmology and Otolaryngology, 75:1333-1345, 1971.

Albrecht, R.M., Hinsdill, R.D., Sandok, P.L., MacKenzie, A.P. and Sachs, I.B.: A comparative study of the surface morphology of stimulated and unstimulated macrophages, prepared without chemical fixation for scanning electron microscopy. Exptl. Cell Res. 70:230-232, 1971.

MacKenzie, A.P.: Freezing, freeze-drying, and freeze-substitution. In "Scanning electron microscopy/1972. Ed.: 0. Johari, Illinois Institute of Technology, Chicago, Illinois. pp. 274-280, 1972.

Rasmussen, D.H. and MacKenzie, A.P.: Effect of solute on ice-solution interfacial free energy; calculation from measured homogeneous nucleation temperatures. In "Water structure at the water-polymer interface." Ed.: H.H.G. Jellinek, Plenum Publishing Corp., New York, N.Y. pp. 126-145, 1972.

George, R.P., Albrecht, R.M., Raper, K.B., Sachs, I.B. and MacKenzie, A.P.: Scanning electron microscopy of spore germination in Dictyostelium discoideum. J. Bacteriology 112:1383-1386, 1972.

Albrecht, R.M., Orndorff, G.R. and MacKenzie, A.P.: Survival of certain microorganisms subjected to rapid and very rapid freezing on membrane filters. Cryobiology 10:233-239, 1973.

Orndorff, G.R. and MacKenzie, A.P.: The function of the suspending medium during the freeze-drying preservation of *Escherichia coli*. Cryobiology 10:475-487, 1973.

MacKenzie, A.P.: Collapse during freeze-drying — Qualitative and quantitative aspects. In "Freeze-Drying and Advanced Food Technology." Ed.: S.A. Goldblith, L. Rey and W.W. Rothmayr. Academic Press Ltd., London. Pp. 277-307, 1975.

Albrecht, R.M. and MacKenzie, A.P.: Tissue cultured and free living cells. In "Principles and Techniques of Scanning Electron Microscopy, Vol. 3." Ed.: M.A. Hayat, Van Nostrand Reinhold Co., N.Y. Pp.109-153, 1975.

MacKenzie, A.P.: The physico-chemical environment during the freezing and thawing of biological materials. In "Water Relations in Foods." Ed.: R.B. Duckworth, Academic Press, London. Pp. 477-503, 1975.

Rapatz, G., Luyet, B. and MacKenzie, A.: Effect of cooling and rewarming rates on glycerolated human erythrocytes. Cryobiology 12:293-308, 1975.

Rasmussen, D.H., Macaulay, M.N. and MacKenzie, A.P.: Supercooling and nucleation of ice in single cells. Cryobiology 12:328-339, 1975.

MacKenzie, A.P., Kuster, T.A. and Luyet, B.J.: Freeze-fixation at high sub-zero temperatures. Cryobiology 12:427-439, 1975.

MacKenzie, A.P.: Principles of freeze-drying. Transplantation Proc., No. 2 (Suppl. 1): 181-188, 1976.

MacKenzie, A.P.: Physico-chemical basis for the freeze-drying process. In: "Developments in Biological Standardization", Vol. 36. Ed.. V.J. Cabasso and R.H. Regamey, S. Karger, Basel. Pp. 51-67, 1977.

MacKenzie, A.P.: Comparative studies on the freeze-drying survival of various bacteria: Gram type, suspending medium, and freezing rate. In: "Developments in Biological Standardization", Vol. 36. Ed.: V.J. Cabasso and R.H. Regamey, S. Karger, Basel. Pp. 263-277, 1977.

MacKenzie, A.P.: Solvent exchange and removal - Lyophilization. In: "Proceedings of the NHLBI Workshop on Technology for Protein Separation and Improvement of Blood Plasma Fractionation." DHEW Publication No. (NIH) 78-1422, pp. 185—201, 1978.

MacKenzie, A.P.: Modeling the ultra-rapid freezing of cells and tissues. In: "Microprobe Analysis of Biological Systems." Ed.: T.E. Hutchinson and A.P. Somlyo. Academic Press, New York. Pp. 397-421, 1981.

MacKenzie, A.P.: Simultaneous DTA and determination of electrical resistance of aqueous solutions during freezing and thawing. In: Proceedings of the 17th. NATAS Conference. Ed.: C.M. Earnest. North American Thermal Analysis Society, Rome, GA, pp. 9-14, 1988.

MacKenzie, A.P.: Freeze-drying of aqueous solutions containing peptides and proteins. In: Therapeutic Peptides and Proteins: Formulation, Delivery and Targeting. Eds.: D.R. Marshak and D.T. Liu. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Pp. 17-21, 1989.

D. Nochlin, A. P. MacKenzie, E. M. Bryant, I. H. Norwood, and S. M. Sumi. 1993, A Simple Method of Rapid Freezing Adequately Preserves Brain Tissue for Immunocytochemistry, Light and Electron Microscopic Examination. Acta Neuropathologica, 86, 645-650.

D. M. Strong and A. P. MacKenzie.1993. Freeze-Drying of Tissues. In: Musculoskeletal Tissue Banking, W. W. Tomford, Editor, Raven Press, New York, Pp. 181-208.

K. R. Morris, S. A. Evans, A. P. MacKenzie, D. Scheule, and N. G. Lordi. 1994. Prediction of Lyophile Collapse Temperature by Dielectric Analysis. PDA Journal of Pharmaceutical Science & Technology, 48:318-329.

S. A. Evans, K. R. Morris, A. P. MacKenzie, and N. G. Lordi. 1995. Dielectric Characterization of Thermodynamic First Order Events in Model Frozen Systems Intended for Lyophilization. PDA Journal of Pharmaceutical Science & Technology, 49:2-8.

W.R. Gombotz, S. C. Pankey, L. S. Bouchard, D. H. Phan, and A. P. MacKenzie. 1996.Stability, Characterization, Formulation, and Delivery System Development for transforming Growth Factor-Beta. In: Formulation, Characterization, and Stability of Protein Drugs, R. Pearlman and Y. J. Wang, Editors, Plenum Press, New York, pp. 219-245.

The following is a brief, but accurate, synopsis of my relevant experience:

Dr. MacKenzie began his continuous career in freezing and freeze-drying in 1959. His earliest studies centered on the fundamental aspects of the freezing and freeze-drying processes. Later his work focused also on pharmaceutical, microbiological and diagnostic applications. Dr. MacKenzie has published more than 50 papers on lyophilization and presented more than 100 papers

from the platform at national and international meetings. Dr. MacKenzie has developed and currently maintains a practical and theoretical interest in the biophysics and hydration of peptides and proteins and their freezing and freeze-drying behavior.

I have read and understood the specification and claims of the subject application, the Office Action dated July 19, 2000, and the Office Action dated September 20, 2001;

and, being as duly qualified, do further declare:

I. The present invention is based on the discovery that Factor VIII compositions can be prepared in a stable dried form by freeze-drying an aqueous solution of Factor VIII using trehalose as a stabilising agent in the absence of albumin.

II. I have been asked to review an issued United States patent to determine its relevance to the present invention. Specifically, I have reviewed the following reference:

Livesey *et al.* (U.S. Patent No. 5,364,756)

III. I have carefully reviewed this reference and, for the reasons discussed below, conclude that there is no teaching or suggestion in this reference of the present invention.

IV. My review of this reference has been done in the context of what was known in the art when the present invention was made. My knowledge in this regard is based on many years of experience in this field dating back to 1959. In 1991, various Factor VIII compositions were known. Factor VIII preparations which were derived from human blood necessarily contained albumin. Although there were significant health risks associated with administering albumin to a patient, namely the potential risk for viral contaminants to be present, the presence of albumin was believed to be necessary in order to stabilise the Factor VIII protein. Recombinant Factor VIII preparations were also being prepared, but again it was believed to be necessary to add albumin to the preparations to stabilise the proteins. The

presence of albumin was believed to be necessary because Factor VIII proteins are extremely labile, even in the presence of other stabilisers.

V. The teachings of the cited reference are entirely consistent with what was generally believed in the art at that time — that albumin was necessary to stabilise Factor VIII compositions. Although the presence or absence of albumin in a Factor VIII composition was clearly not the main focus of this reference, a careful review of this reference reveals evidence which supports the proposition that one skilled in the art would not have expected to be able to freeze dry Factor VIII in the absence of albumin.

VI. The Livesey *et al.* patent describes the cryopreservation of biological materials. The method and apparatus described by Livesey *et al.* can purportedly be used for cryopreservation of materials ranging from viruses to cultured mammalian cells. However, it is apparent from the disclosure, including the examples, that the primary focus is on the preservation of whole cells. Red blood cells, plateletes, leukocytes, sperm, pancreatic islets, and marrow cells are all listed as specific examples of cells which can be preserved using the Livesey *et al.* procedures. There is very little discussion, and there are no examples, of the preservation of proteins. Those skilled in the art know that materials and procedures used to preserve whole cells and/or viruses are not necessarily applicable to the stabilisation of proteins. Furthermore, Livesey *et al.* state that the exact ingredients of the suspensions which are to be preserved "is not considered to be a component of the invention." Thus, I find no disclosure in the Livesey *et al.* patent which is specifically relevant to the selection of appropriate stabilising agents for delicate proteins.

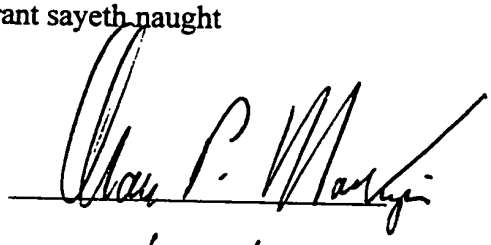
VII. Freeze drying of biological materials is a very complex, and poorly understood, process. Livesey *et al's.* extensive discussion of this process contains much information which is not relevant to the current review. However, the critical differences between ambient drying and cold temperature drying are expressly acknowledged by Liversey *et al.* It

is clear from the discussion in the Livesey *et al.* reference that protection of proteins from damage due to freezing involves very different mechanisms from protecting proteins from damage due to drying. Livesey *et al.* contrast the role of cryoprotectants with dry protectants stating that "[t]he cryosolution may also include exposing the biological suspension to one or more dry protectant compounds. Dry protectants, by definition, stabilize samples in the dry state." Livesey *et al.* specifically list human serum albumin as a cryoprotectant which has been found to be effective in combination with trehalose (a dry protectant). Thus, the Livesey *et al.* reference does not teach that trehalose can be used in the absence of albumin.

VIII. Trehalose is only mentioned by Livesey *et al.* as one possible ingredient in a drying process. There is no reference to preparing a Factor VIII composition in the absence of albumin, and it cannot be inferred, given the knowledge at that time, that albumin was not required. Thus, the Livesey *et al.* discussion of stabilising agents is entirely consistent with the proposition that Factor VIII cannot be freeze-dried without the use of albumin as a stabilising agent. In my opinion, there is nothing in the Livesey *et al.* specification or claims which teaches, or even suggests, to those skilled in the art, that Factor VIII can be freeze-dried in the absence of albumin.

The undersigned declares further that all statements made herein of his own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the Application or any Patent issuing thereon.

Further declarant sayeth naught

Signed: 

Date: 10/18/01

EXHIBIT A

Products currently available for use in hemophilia A

Cryoprecipitate. Prepared from single plasma units by cold precipitation and resuspension in plasma. Although the source plasma is screened for HIV 1 and 2, anti-HBc, ALT, and anti-HCV, there is no viral inactivation step for the cryoprecipitate normally obtained from your local blood bank. Because of the theoretical risk of HIV infection, the use of cryoprecipitate to treat hemophilia is not recommended. The New York Blood Center is currently working on techniques to treat cryoprecipitate using solvent-detergent techniques but this is not currently available.

Intermediate purity factor VIII. Prepared from pooled human plasma which is screened for anti-HIV 1 and 2, ALT, anti-HBc, anti-HTLV I/II, and anti-HCV. Viral inactivation steps are used in all intermediate purity concentrates although the techniques differ from product to product. These inactivation steps generally appear to be highly effective against HIV and selected hepatitis viruses. The U. S. Hemophilia/HIV Seroconversion Surveillance Project sponsored jointly by the Centers for Disease Control, the Food and Drug Administration, and the National Hemophilia Foundation has shown no HIV seroconversions in hemophilia attributable to factor concentrates since 1987 and no hepatitis B or C seroconversions in hemophilia attributable to factor concentrates since 1992.

Intermediate purity factor VIII products which are currently available:

- **Factor VIII SD** (NY Blood Center; Melville) - Viral inactivation by extraction with TNBP - sodium cholate.
- **Humate-P** (Behringwerke) - Pasturized product prepared by heat treatment in solution to 60°C for 10 hours.
- **Profilate OSD** (Alpha) - Viral inactivation using TNBP - polysorbate 80.
- **Koate HP** (Bayer-Miles) - Viral inactivation using TNBP - polysorbate 80.
- **MelATE** (Melville) - Viral inactivation using TNBP - polysorbate 80.
- **Alphanate** (Alpha) - Purified from plasma by affinity chromatography using heparin-agarose to bind von Willebrand factor which is complexed with factor VIII. Viral inactivation using TNBP - polysorbate 80.

Monoclonal antibody purified factor VIII. Prepared from pooled human plasma which is screened for anti-HIV 1 and 2, anti-HBc, ALT, anti-HTLV I/II, and anti-HCV. The factor VIII is purified by affinity chromatography using mouse monoclonal antibodies to human factor VIII. The purified factor VIII prior to formulation has a specific activity of ~3000 units per mg. Human albumin is used as a stabilizer in the formulation of the factor VIII.

Monoclonal factor VIII products which are currently available:

- **Monoclate-P** (Centeon) - Prepared from commercial source plasma.
- **Hemofil-M** (Baxter) - Prepared from commercial source plasma. Affinity purified using a mouse monoclonal antibody which recognizes the factor VIII heavy chain.
- **AHF-ARC** (Red Cross) - Prepared from Red Cross volunteer source plasma and purified and formulated at Baxter using the same methods used to purify Hemofil-M.

Recombinant factor VIII. Recombinant factor VIII is a synthetic form of factor VIII prepared in mammalian cells, such as Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells. These are immortalized cell lines which are stable in culture, and permit the high level expression of human proteins with expected post-translational modifications such as tyrosine sulfation and carbohydrate attachment. Initial concerns about the possible immunogenicity of recombinant factor VIII have been somewhat alleviated by 1) studies in previously-treated patients showing a very low prevalence of inhibitors, 2) studies in previously-untreated patients (PUPs) showing a higher but stable prevalence of inhibitors, and 3) studies in newborn and transgenic mice showing a lack of immunogenicity of recombinant factor VIII compared with plasma factor VIII. Because both products are formulated with human albumin, even though the albumin is pasturized, recombinant factor VIII is subject to the same recalls as plasma-derived factor VIII in the event that donors with Creutzfeld-Jacob disease contribute to the source plasma from which the albumin is prepared. Two forms of recombinant factor VIII are currently licensed for use.

Recombinant factor VIII products which are currently available:

- **Recombinate** (Baxter) - The full length cDNA for factor VIII is used to synthesize Recombinate. Synthesis is in Chinese hamster ovary (CHO) cells and the factor VIII is purified by monoclonal affinity affinity chromatography using a mouse anti-factor VIII antibody which recognizes the 90 kDa heavy chain of factor VIII. The purified factor VIII is formulated with pasturized human albumin as a stabilizer. No viral inactivation step is used in Recombinate. Licensed by the FDA in December 1992.

- **Kogenate** (Bayer-Miles) - The full length cDNA for factor VIII is synthesized in baby hamster kidney (BHK) cells, purified by monoclonal affinity chromatography using a mouse anti-factor VIII antibody that recognizes the x domain of factor VIII, and formulated with pasturized human albumin. No viral inactivation step is used in Kogenate. Licensed by the FDA in March 1993.

- **Bioclate** (Centeon) - identical with Baxter Recombinate and distributed by Centeon under a licensing agreement on the patent for factor VIII.

- **Helixate** (Centeon) - identical with Bayer-Miles Kogenate and distributed by Centeon under a licensing agreement on the patent for factor VIII.

The Medical and Scientific Advisory Committee (MASAC) recommends that individuals with hemophilia and their providers should consider the use of recombinant derived clotting factor as the first choice for replacement therapy.

Click here for more information on product safety and product recalls from the Food and Drug Administration.

Click here to return to the UNC Hemophilia Center Home Page.

EXHIBIT B

Recombinant Protein Drugs
ed. by P. Stuckel
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Clotting factors VIII and IX

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[2] *Oxford Haemophilia Centre, The Churchill Hospital, Oxford, UK*

Introduction

Haemophilia is the most common congenital disorder of coagulation and affects approximately 1 in 10,000 males around the world. Haemophilia A is due to a deficiency of factor VIII in the circulating blood whilst haemophilia B (also known as Christmas disease) is a clinically identical disorder caused by factor IX deficiency. It is less common than haemophilia A and affects 1 in about 30,000 males. Both factors VIII and IX are essential glycoproteins in the clotting cascade [1] (Fig. 1). The hallmark of severe haemophilia is recurrent and spontaneous haemarthrosis, typically affecting the hinge joints such as the ankle, knee and elbow. The severity of bleeding depends upon the level of factor in the blood.

Severe haemophilia is usually defined by a level of <2 iu/dl (or <2%) of factor VIII or IX in plasma. Moderately severely affected patients have levels varying from 2–5 iu/dl and mild from 5–25 iu/dl. It is unusual for an infant to have spontaneous haemarthroses in the first few months of life, and the first joint to be affected tends to be the ankle as the child learns to crawl. Repeated bleeding into joints may cause permanent damage, with painful arthritis and limb deformity and associated muscle wasting. Bleeding into muscles is also a feature of haemophilia, but this is usually a consequence of direct injury, albeit often minor. Bleeds into certain areas are particularly dangerous because of the risk of compression of neighbouring structures. Bleeds in the tongue can obstruct the airway, and retroperitoneal bleeding within the ilio-psoas muscle may result in femoral nerve compression. Bleeding from the gastrointestinal tract and bleeding into the urinary tract may also occur. There is also a significant risk of intracranial haemorrhage in severe haemophilia which was a significant cause of mortality in the past when treatment was not so readily available. The poor prognosis in the absence of effective treatment is reflected by the fact that the median life expectancy of haemophiliacs in Sweden was only 11 years during the period 1831–1920, but rose to 56.8 years during the period 1961–1980 [2]. It is also interesting to note that a century ago haemophilia affected several members of the royal families of Britain, Spain and Russia,

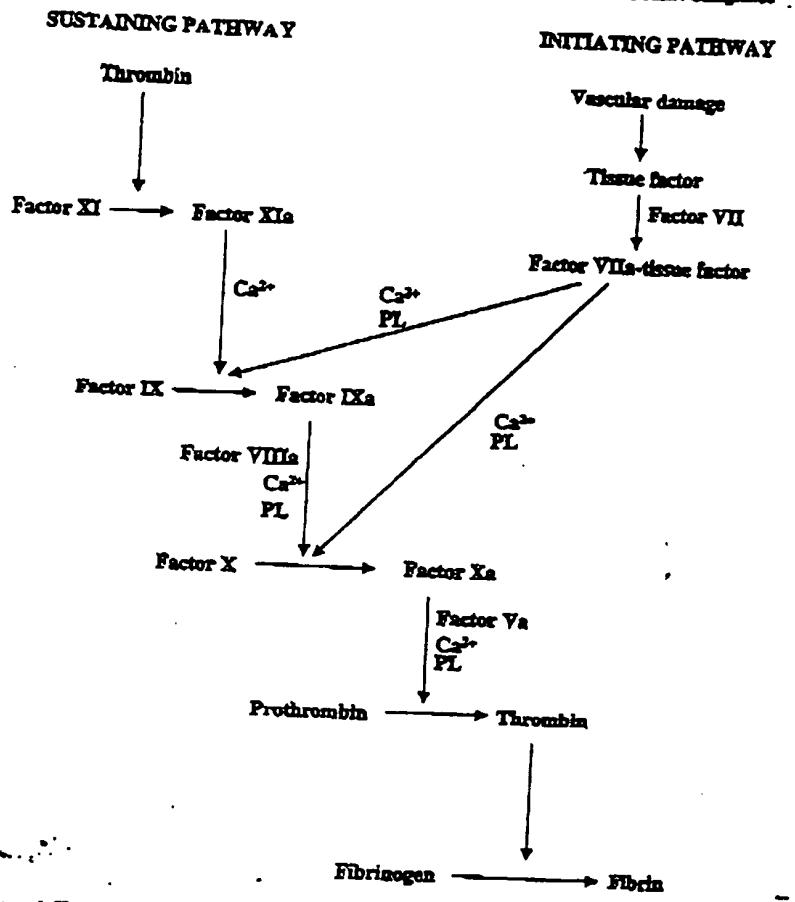

Figure 1. The revised clotting cascade. Thrombin, formed by the action of factor Xa as a result of vascular damage in the initiating (or extrinsic) pathway activates factor XI, thereby sustaining the cascade through the sustaining (or intrinsic) pathway. PL = phospholipid. The scheme is simplified and omits many details for clarity [1].

but, despite the best medical facilities of the age being available, all affected members died in their youth and no surviving descendants are affected.

Approximately 5% of patients with haemophilia A develop inhibitory antibodies to factor VIII at some stage but it is quite likely that this figure underestimates the true prevalence. Inhibitor development in haemophilia B is, by contrast, very rare (<1%). This is a potentially serious complication of therapy, as patients are refractory to conventional doses of coagulation factor concentrates and bleeding can be difficult to control. Family studies suggest that

there is a genetic predisposition to the formation of antibodies but no HLA association or other linkages have been conclusively identified. There is some evidence that people of Afro-Caribbean origin are more susceptible to inhibitor formation than other ethnic groups. Certain mutations are undoubtedly associated with a significantly increased risk of inhibitor development, particularly large gene deletions and nonsense mutations resulting in stop codons – presumably because patients lack immune tolerance [3, 4]. There is a weaker association between inhibitor formation and the presence of an inversion in intron 22 of the factor VIII gene [5].

The genes for factor VIII and IX are both located at the telomere of the long arm of the X chromosome and thus haemophilia is inherited as an X-linked, recessive condition affecting males. The daughters of affected males are obligate carriers but sons are normal. A proportion of all cases of haemophilia occurs in the absence of a previous family history and is due to new mutations. Perhaps the most famous example is that of Queen Victoria, who had a haemophiliac son (Leopold) and also two daughters who were carriers. It is not known, however, whether they suffered from haemophilia A or B. There are very rare instances of haemophilia affecting females due to inheritance of the defective gene from both parents or to unequal X-inactivation in carriers [6]. There are also reports of haemophilia in females with Turner's syndrome (XO karyotype) and congenital androgen insensitivity (testicular feminization with XY karyotype).

Molecular basis of haemophilia A

Factor VIIIa is an essential cofactor which is required for the activation of factor X by factor IXa in the clotting cascade (Fig. 1). Factor VIII is a glycoprotein of 2332 amino acids and is synthesized predominantly by hepatocytes of the liver. It is processed intracellularly in the Golgi apparatus by proteolytic cleavage giving rise to a N-terminal heavy chain and a C-terminal light chain. Its domain structure (Fig. 2A) includes a carbohydrate-rich B domain that is not required for activity (see below). Factor VIII is activated to give rise to factor VIIIa by further proteolytic cleavage, probably by thrombin. In plasma, factor VIII circulates as a large glycoprotein complex non-covalently bound to multimers of von Willebrand factor (Fig. 2). The factor VIII gene is about 186 kb in length, with 26 exons, and is situated on the long arm of the X chromosome at Xq28.

Developments in molecular biology have permitted rapid identification of mutations in haemophilia A (and B) patients by various methods. PCR amplification of either genomic DNA or cDNA derived from the reverse transcription of mRNA of patient lymphocytes has superseded older methods based on restriction enzyme digestion and Southern blotting. Although automated DNA sequence analysers have been developed, gene sequencing of the entire factor VIII gene would be both expensive and labour-intensive because of its size.

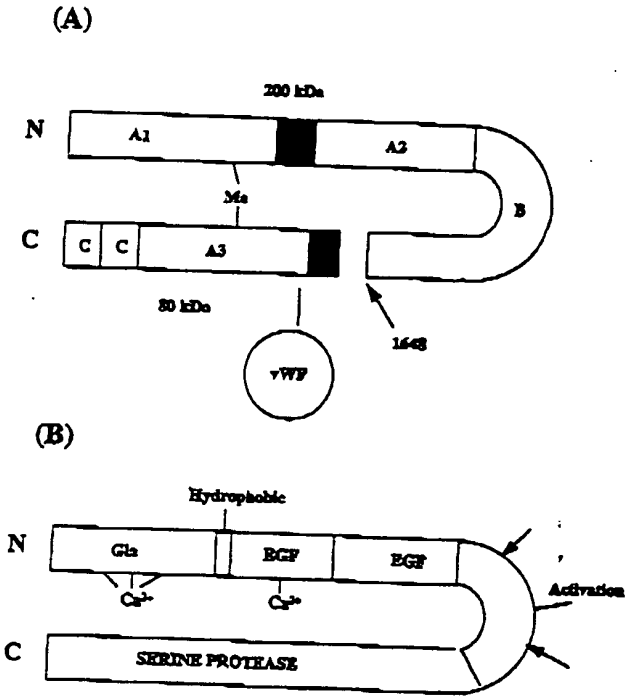

Figure 2. The domain structure of (A) factor VIII and (B) factor IX. In factor VIII (above) the N-terminal heavy chain and C-terminal light chain, formed after proteolysis at amino acid 1648 as shown, are stabilized by a metal (Me) binding site; a proposed site of binding to von Willebrand (vWF) factor is shown. The A1 and 2, B and C domains are marked. In factor IX (below) arrows mark the sites of proteolytic cleavage in the activation domain by either factor XIa or VIIa. For further details, see text.

Thus methods have been developed to initially prescreen the gene or mRNA, in order to define the mutated exon of the DNA, or region of the mRNA, before sequencing to define the actual mutation itself. Chemical cleavage of mismatches in heteroduplexes formed from the mutated DNA and a test DNA is the best current technique for detecting all mutations [7, 8]. Other useful methods are conformation-sensitive gel electrophoresis and denaturing-gradient gel electrophoresis, although these alternatives do not allow all mutations to be detected.

By far the commonest single genetic defect causing haemophilia A is an inversion in intron 22, which is encountered in about 20% of all patients, and in nearly half of all severely affected patients [5]. The inversion mechanism proposed involves homologous, intra-chromosomal recombination between an intronless gene of unknown function, designated F8A, which lies within intron

22 of the factor VIII gene and either of 2 further copies of F8A flanking the factor VIII gene. In either case, the inversion results in a truncated factor VIII gene generating a truncated mRNA and inactive factor VIII protein, thus explaining the severe haemophilia. Recombination with the distal copy of F8A is commoner than with the proximal copy and accounts for approximately 80% of the inversions (reviewed in [9]). It is now recognised that inversion is much more common in a male than a female meiosis.

All other known haemophilia A mutations are summarized in a valuable mutation database [10]. At present, 454 unique mutations are listed, including 92 cases of large deletions. However, it is generally thought that large deletions account for only a minority of cases of haemophilia and the reason that a much higher proportion than 5% is listed in the database reflects an ascertainment bias. Most mutations are point mutants or very short additions or deletions of only a few nucleotides. Nonsense, frameshift and splice-site mutations, caused by point mutations or short additions or deletions, usually cause severe haemophilia. Most other cases are caused by missense mutations causing amino acid substitutions and 218 missense mutations are presently known [10]. They have varying degrees of severity for patients from very mild to very severe [10]. Mutations at CG doublets, giving rise to a TG or CA doublet, are common. As is well known, CG doublets are genomic "hotspots" because of specific methylation of cytosine to 5' methylcytosine and because the methylated cytosine is particularly susceptible to mutation by deamination.

Molecular basis of haemophilia B

Factor IXa is a serine protease required for the activation of factor X in the clotting cascade. Factor IXa is itself formed from factor IX by proteolysis by either factor VIIa-tissue factor complex, which initiates the clotting cascade, or by factor XIa, which sustains it [1] (Fig. 1). Factor IX is a glycoprotein of 415 amino acids and is made up of a N-terminal γ-carboxyglutamic acid-rich sequence (Gla domain), two epidermal growth factor-like (EGF) domains – the first of which binds calcium – an activation domain and finally a C-terminal serine protease or catalytic domain (Fig. 2B). The 12 glutamic acid residues of the Gla domain undergo γ-carboxylation by a vitamin K-dependent γ-glutamyl carboxylase in the endoplasmic reticulum (ER) during the synthesis of factor IX in the liver hepatocyte. This essential post-translational modification is necessary for the correct protein folding and calcium binding of factor IX. The factor IX gene is about 34 kb in length and contains eight exons. Its basic exon structure is similar in organisation to coagulation factors VII, X and protein C and it is likely that they all evolved from a common ancestral gene by gene duplication.

The factor IX gene is considerably smaller than that of factor VIII, and patients with haemophilia B have been studied more extensively than those with haemophilia A. The first defects identified in haemophilia B were large

gene deletions detected in patients with inhibitory antibodies by Southern blotting [3]. However, it is now clear that point mutations, or short additions or deletions, account for the vast majority of cases of haemophilia B, and over 689 unique mutations are listed in an extensive mutation database of 1918 families who have been studied from around the world [6, 11]. Most of the unique mutations are point mutants causing missense, nonsense or splicing defects involving all 8 exons. For example, there are at present 425 different missense mutations that cause amino acid substitutions. These include the original case of Christmas disease, which is a G → C mutation at nucleotide 30,070 causing an amino acid change from Cys → Ser at residue 206 within exon 7 [12]. Many mutations have been observed more than once, even where there is no known kinship. Many of these repeat mutations – as in the case of haemophilia A patients, occur at CG doublets, suggesting they are independent mutation hotspots. However, other repeat mutations do not involve CG doublets, suggesting a founder effect. A good example is the mutation at nucleotide 31,311 where there are 41 examples.

A few patients have been described in whom the factor IX level rises significantly after puberty (the haemophilia B Leiden patients); this is associated with complete clinical recovery. These interesting regulatory mutations have been all localized to a short region of the factor IX promoter and 18 unique mutations are now known [6]. These mutations inhibit the binding of transcription factors, e.g., hepatic nuclear factor 4, which are required for the efficient initiation of transcription. An androgen response element (ARE) in the factor IX promoter, which interacts with the androgen receptor in the presence of testosterone, is responsible for the upregulation of factor IX mRNA at puberty [13]. Consistent with this hypothesis, patients with mutations in the ARE do not recover at puberty [13].

Treatment of haemophilia

Over a century ago, the first effective product available for treatment of bleeding episodes was fresh blood. The preparation of an antihaemophilic factor of bovine origin by Macfarlane in 1954 [14] was a major therapeutic advance, although serious allergic reactions were not infrequent. The use of cold-insoluble cryoprecipitate was introduced in 1965. The subsequent development of lyophilized concentrates of factors VIII and IX in the early 1970s transformed the life of haemophiliacs. The goals of treatment, as stated by the World Federation of Haemophilia, are "to minimise disability and prolong life, to facilitate general social and physical well-being and to help each patient achieve full potential whilst causing no harm" (www.wfh.org). Advances in therapy have certainly resulted in a dramatic increase in the longevity of haemophiliacs in developed countries. A study of 717 Dutch patients documented a calculated life expectancy for these patients of 66 years, compared with 74 year for normal males [15], and the authors of this study concluded

that the mortality associated with even severe haemophilia was similar to that associated with cigarette smoking.

Treatment of bleeding episodes involves the intravenous injection of coagulation factor concentrates. The total dose and frequency of treatment is determined by the severity and site of bleeding. Most bleeds resolve with a single infusion, if the bleed is recognised early and treated promptly. There is an increasing move to prophylactic therapy, in which the patient gives himself injections at home of coagulation factors two or three times a week to prevent bleeds rather than just treating on demand when bleeds occur. Patients on prophylactic therapy experience few or even no spontaneous bleeds and thus progressive joint damage and arthritis can be avoided.

The pooling of plasma donations introduced the risk of transmission of viral infections, and many haemophiliacs were infected with HIV and/or hepatitis C in the 1980s. In the UK, 1229 haemophiliacs were infected with HIV, and about 300 were exposed to hepatitis C [16, 17]. The introduction in 1985 of physical methods of viral inactivation, such as heat-treatment or the addition of a solvent/detergent mixture, eliminated the risk of transmission of HIV or hepatitis C. However, other viruses are relatively resistant to these measures and cases of hepatitis A and parvovirus infection have been documented even with these more modern products. More recently, there has been concern about the possibility of transmission of prion disorders, although no cases have yet been described in haemophilia [18]. Recombinant products offer the greatest margin of safety for haemophiliac patients and have been recommended as the treatment of choice for all patients with haemophilia (see below).

Development of recombinant DNA methods

During the late 1970s there was a quiet revolution in molecular biology. Using restriction enzymes and DNA ligase, it became possible to clone copies of mRNA into either bacterial plasmids or into bacteriophages such as phage λ. Such clones could then be sequenced using Sanger's dideoxy chain termination method or Maxam & Gilbert's chemical degradation procedure. Once these mRNAs had been cloned, each cloned cDNA served as a probe in order to isolate from the entire genome its respective gene, which could then in turn be sequenced. Oligonucleotides were starting to be used as primers for the enzymatic synthesis of DNA. The presence of introns in most genes was discovered. Southern had described his blotting technique that was to become so widely used in this field until the development of PCR techniques much later in the 1980s.

Globin, immunoglobulin and ovalbumin mRNA – all mRNA species which could be purified in reasonable quantities from specialized cells or organs – could now be cloned in *E. coli* and sequenced in their entirety for the first time. Moreover, the new cloning methods suggested that mRNA and/or genes of medical interest, such as human insulin and human growth hormone mRNA,

might be cloned and the protein expressed artificially in bacteria. This, it was thought, might be a cost-effective and safer alternative to isolating these proteins from natural sources for the treatment of diabetes and pituitary dwarfism.

1980 was a time of optimism and some of us thought that we might be able to clone rare, low-abundance mRNA, if only new methods could be developed. We decided to clone human factor IX mRNA. This would be an ambitious and difficult project because of its low abundance in liver. However, if the project were successful, the benefits for patients with haemophilia B would be considerable.

Recombinant factor IX

The factor IX gene was cloned in 1982 – some 2 years before the factor VIII gene – and this work will be described first. The expression of biologically active recombinant factor VIII, however, proved technically easier than factor IX. Moreover the priority was to produce recombinant factor VIII before recombinant factor IX because of the higher incidence of haemophilia A than B in the population. Consequently recombinant factor VIII was available for the treatment of patients in 1992, much sooner than recombinant factor IX, which was only produced much later in 1997.

Cloning the human factor IX gene in Oxford

It was the availability of the complete amino acid sequence of bovine factor IX [19] that initially led us to think that it might be possible to clone the factor IX gene. We thought that short synthetic oligonucleotides, with their sequence partly predicted from the genetic code and the amino acid sequence of the protein, would probably be the key to cloning rare mRNAs, like the factor IX mRNA. We had previously used oligonucleotides both for our earlier sequencing of regions within the α and β-globin, ovalbumin and immunoglobulin mRNAs [20], as well as for our later influenza cloning [21, 22]. Oligonucleotides were clearly powerful and highly specific reagents for nucleic acids. In particular, they could be used to hybridize to specific sequences in mRNA and prime the synthesis of cDNA by reverse transcriptase.

We first decided to enrich the bovine factor IX mRNA, which we had isolated from a calf liver, by two successive sucrose density-gradient centrifugation steps [23]. We then set up a rabbit reticulocyte *in vitro* translation assay to detect factor IX mRNA, locating it in the 20–22S fraction of the sucrose gradient. We estimated that it had been purified about 10-fold over the starting mRNA. Even so it was unlikely that the factor IX mRNA was >0.1% pure at this stage.

We then synthesized, as a mixture, eight 14-nucleotide (nt) long oligonucleotides (oligo N1 mixture), complementary to the bovine mRNA sequence, whose sequence was predicted from the amino acid sequence of residues 348–352 of bovine factor IX (Fig. 3). This was a particularly favourable region

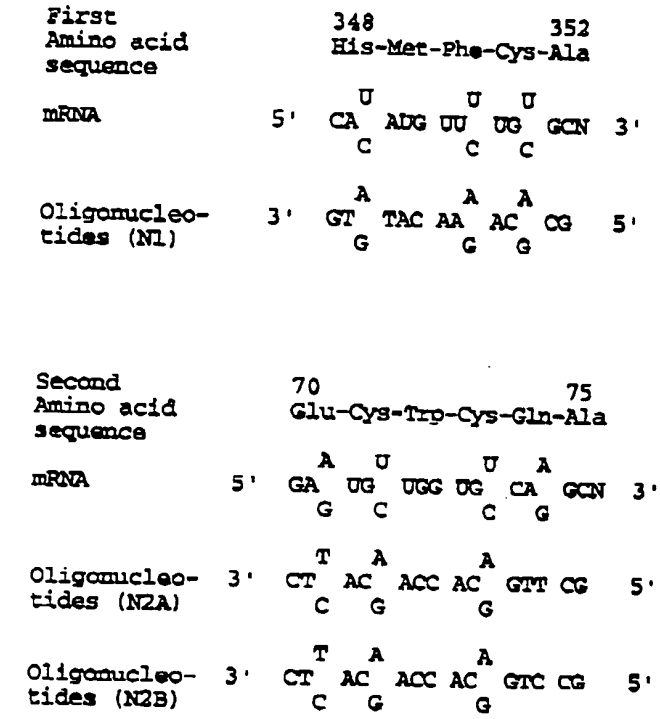

Figure 3. Two regions of amino acid sequence of bovine factor IX used to design mixed oligonucleotide sequence primers (N1) or probes (N2A & N2B) [22]. See text for further details.

of amino-acid sequence since the number of alternative possible nucleotide sequences predicted from the genetic code was limited to 8, because the sequence contains a methionine residue which has a unique codon. This oligo N1 mixture of primers was used to prime the synthesis of cDNA from factor IX-enriched bovine mRNA in order to generate a library of cDNA clones. Briefly, after synthesis of cDNA by reverse transcriptase and subsequent removal of mRNA by alkaline hydrolysis, "loop-back" synthesis of double-stranded DNA was catalysed by *E. coli* DNA polymerase I (Klenow subfragment) in the absence of added primer. Cloning of this double-stranded DNA was simplified by restriction digestion of DNA with MboI (GATC-recognition sequence) and ligation into the unique Bam HI site of the classic plasmid, pBR322. An "MboI" cDNA library of about 7000 recombinant clones was thus obtained in *E. coli*.

Bacterial colonies were then screened on Whatman 541 paper [24] using a second mixture (oligo N2A plus oligo N2B) of 16 ^{32}P-labelled 17-nt long

oligonucleotides based on the predicted sequence of bovine factor IX mRNA of another favourable region of amino acid sequence between residues 70–75 (Fig. 3). We obtained a single clone, labelled BIX-1, which was sequenced by the Maxam and Gilbert method. This clone encoded bovine factor IX mRNA from amino acids 52 to 139 and predicted an amino acid sequence that was in complete agreement with the literature, except for a single discrepancy changing the amino acid at position 57. This discrepancy was, in fact, confirmed as a correction to the published bovine factor IX sequence, since it was also present in an independent clone in a "dC/dG-tailed" cDNA library of about 10,000 *E. coli* colonies.

Thus the choice of oligonucleotides and our ability to synthesize them in-house were two important factors in our initial success in isolating factor IX cDNA clones. Acting in the hope that the human and bovine factor IX genes would be sufficiently conserved in nucleotide sequence to cross-hybridize, the bovine factor IX probe was then used to probe a Charon 4A bacteriophage λ library of human genomic clones [22]. A positively hybridizing clone, λHIX1b, was isolated and mapped by restriction enzyme cleavage and Southern blotting. A restriction fragment corresponding to the probe was thus identified on Southern blots and sequenced. There was 85% nucleotide sequence conservation between bovine and human factor IX gene in the region initially sequenced. We had thus isolated a clone containing part of the human factor IX gene.

Further cloning and expression of factor IX in mammalian cells

Further cloning Two additional reports from other groups [25, 26] extended our initial work by describing the complete coding sequence of human factor IX, which was found to be preceded by a leader sequence with a potential signal peptide and propeptide sequence. There was uncertainty, however, as to whether the entire coding sequence had been cloned, since the 5' non-coding of the factor IX mRNA was not characterized. There were, also, in one report [26] some 6 differences in the coding nucleotide sequence attributed, incorrectly as it turned out later except for one nucleotide [27], to the presence of polymorphisms. Interestingly, although the details of the cloning procedures differed in these reports from our own work, all of the studies had relied on oligonucleotides to prepare or isolate clones from cDNA libraries.

We concentrated on characterizing the complete factor IX mRNA sequence by cDNA cloning, and independently sequencing all exons in order to define splice points and to check for potential cDNA cloning errors, which are known to occur during reverse transcription and cloning. We defined the mRNA start site precisely and sequenced the entire 3' non-coding region of the mRNA [27]. The factor IX gene turned out to be about 34 kb long with 8 exons. The factor IX mRNA was about 2800 nucleotides long and had a relatively short 5' non-coding region, but a reasonably long 3' non-coding sequence of nearly 1400 nucleotides containing the usual AAUAAA poly(A) signal. Finally, the complete human factor IX gene sequence of 33.5 kb, including all 7 introns, was established by Davie's group [28].

Expression of biologically active factor IX in mammalian cells If cloning the factor IX cDNA and gene sequence were to be a "useful" contribution to medicine, then expression of recombinant factor IX protein would have to be achieved. A major uncertainty was whether *biologically active* factor IX was synthesized, because after synthesis, factor IX has to be post-translationally modified and correctly processed. In particular, after γ-carboxylation of 12 glutamyl residues near the N-terminus of factor IX by a vitamin K-dependent carboxylase, the 18 amino acid residues of the propeptide sequence [29] must be cleaved by a furin protease. Both these processes are essential for factor IX activity. It was conceivable that these essential post-translational events would only occur in liver, the site of factor IX synthesis in the body, and would not occur in hepatic or other cell types derived from other tissues in tissue culture.

We were fortunate that the aim of producing biologically active factor IX in mammalian cells in tissue culture was feasible but it was only achieved after a considerable effort by Don Anson and Ian Jones in the Brownlee laboratory. We published, in 1985, that "we were able to isolate small amounts of biologically active (recombinant) human factor IX" [30]. In particular, factor IX was expressed in rat hepatoma cells that had been stably transfected with a factor IX expression plasmid. Much higher yields of >90% biologically active factor IX were obtained in canine kidney cells (MDCK cells) using a slightly different expression plasmid [31]. Our results were particularly convincing because we purified the recombinant factor IX by immuno-affinity chromatography.

Subsequently, higher yields of recombinant factor IX were achieved by other groups using different expression vectors in different mammalian cells, i.e., baby hamster kidney (BHK), human hepatoma (HepG2) and chinese hamster ovary (CHO) cells [32–34]. But in every case the biological activity of factor IX was less than in our reports. Indeed, in the initial work by Kaufman's group in CHO cells (in which the factor IX gene was amplified in the genome) factor IX was secreted to give as much as 100 mg/ml of factor IX in the medium, yet only 2% was biologically active [34]. This low biological activity in CHO cells was subsequently solved by the introduction of a furin-type protease (see below), suggesting that factor IX propeptide processing must have been limiting.

Development of recombinant factor IX for clinical use

The observation that some, albeit low, biological activity was present in the factor IX expressed in CHO cells was important [34], since this cell line has properties that are particularly suited for large-scale culture needed for the industrial production of recombinant proteins by the biotechnology industry. Therefore, development work was undertaken by Genetics Institute, Boston. This presented a significant challenge since it was essential that propeptide processing and γ-carboxylation, known to be essential for factor IX activity, had to occur. Neither of these processes had been previously required in CHO cells in the production of recombinant proteins for the biotechnology industry.

Initially it was suspected that γ-carboxylation was limiting in the factor IX-expressing CHO cells, thus accounting for the presence of the inactive, incompletely γ-carboxylated factor IX. However, when the cloned γ-carboxylase became available, transient transfection of expression constructs of the γ-carboxylase into factor IX-secreting CHO cells failed to improve factor IX biological activity [35]. By contrast, propeptide processing of factor IX was significantly improved by the co-expression of a furin-like enzyme, specifically a soluble form of PACE (paired basic amino acid cleaving enzyme) introduced stably into the CHO cell line [36]. Finally, Genetics Institute produced factor IX in a form that was fully processed by screening factor IX-expressing CHO clones to identify those with the highest factor IX-processing capacity, by optimizing the concentration of vitamin K added to the defined serum-free culture medium (which lacked any added protein) and by defining the precise conditions of growth of the factor IX-secreting CHO clone in large-scale (2500 l) bioreactors.

A down-stream large-scale purification protocol was developed that did not require the use of monoclonal antibodies for affinity purification, to avoid any risk of introducing viral contaminants. The purified recombinant factor IX showed a slightly reduced γ-carboxylation level of about 11 γ-carboxyglutamyl (Gla) residues per molecule compared to 12 Gla residues in blood-derived factor IX (Tab. 1 and ref. [37]). A new result obtained from the analysis of this recombinant factor IX is that the post-translational modification of Gla residues 36 and 40 are not apparently needed for activity [38]. Other differences between recombinant and human blood-derived factor IX were appar-

Table 1. Comparison of post-translational modifications of plasma-derived and recombinant factor IX

Modification	Plasma-derived	Recombinant
1. γ-carboxyglutamic acid (Gla)*		
12 of 12 Gla residues	100%	60%
11 of 12 Gla residues	0%	35%
10 of 12 Gla residues	0%	5%
2. β-hydroxyaspartic acid 64	37%	46%
3. Carbohydrate		
N-linked glycans		
Asn 157 }	High heterogeneity	Low heterogeneity
Asn 167 }	fully sialylated	<fully sialylated
O-linked glycans		
Ser 53	$(Xyl)_{1-2}$-Glc	$(Xyl)_2$-Glc
Ser 61	NeuAcGalGlcNAcFuc	NeuAcGalGlcNAcFuc
Thr 159, 169 & 172	Classical, partially filled	Classical, partially filled
4. Tyr 155 sulphation	>90%	<15%
5. Ser 158 phosphorylation	>90%	<1%

Footnotes: Adapted from ref. [37] with permission. *The 10 completely modified Gla residues occur at amino acid residues 7, 8, 15, 17, 20, 21, 26, 27, 30 and 33. Gla residues at positions 36 and 40 are incompletely modified, as indicated, in recombinant factor IX.

ent in the extent of N- and O-linked carbohydrate side chains, and in sulphation and phosphorylation levels of particular residues (Tab. 1). The extent of β-hydroxyaspartate modification at serine 64, however, was similar. Fortunately, none of the differences in post-translational modification seemed to affect the biological activity of factor IX, although the differences in sulphation and phosphorylation, it was suggested, may explain the slightly lower recovery (on average about 20% lower) of recombinant, as compared to blood-derived, factor IX seen in patients [37].

In summary, recombinant factor IX is already really a "second-generation" recombinant product because it is prepared from cultured cells and purified under conditions where there is no contact with human or bovine proteins [37]. Thus the product should be free of known blood-borne viral diseases, such as HIV, hepatitis A, B or C and parvoviruses. There should also be no risk from diseases caused by prions, such as Creutzfeldt-Jakob disease (CJD) or the variant CJD, caused by the prion of bovine spongiform encephalitis in cattle. Recombinant factor IX (BeneflX) was approved for sale in the USA and Canada in 1997 and in Europe in 1998.

Recombinant factor VIII

When factor VIII was first cloned and expressed in cultured cells by two competing companies in 1984, this achievement was hailed as one of the most exciting advances to be reported by the biotechnology industry [39]. However, in a Nature editorial in the same week that the factor VIII cloning was announced, we were poignantly reminded that the first deaths had occurred in Australia and in the UK from HIV-contaminated blood. Clearly blood-derived clotting factors were potentially dangerous and would eventually be superseded by recombinant proteins. Not for the first time was the fear of disease driving scientists and biotechnology companies to find improved and safer medicines.

Cloning and expression of factor VIII

The factor VIII gene and its cDNA were cloned by the use of oligonucleotide probes based on the amino acid sequence of peptides isolated from either human or porcine factor VIII, essentially by similar methods to those employed previously to clone factor IX. Due to its low concentrations in plasma, its high molecular weight and extreme sensitivity to proteolytic processing, it was a very difficult protein to purify. The breakthrough came when amino acid sequence information on peptide fragments of human or porcine factor VIII became available [40, 41].

Surprisingly, a unique 36-nt long oligonucleotide, rather than a mixture of oligonucleotides, was successfully used as a probe in one report [40], and in the other [41] two sets of mixed oligonucleotides, either 45-nt long or 15-nt long, were used. Because of uncertainty as to whether factor VIII was synthe-

sized in the liver, both groups initially cloned the factor VIII gene by screening genomic libraries of clones in bacteriophage λ with these probes. The gene turned out to be 180 kb long with 26 exons and at that time was the longest known gene. Human cDNA clones were then subsequently isolated, using genomic probes, by screening appropriate human cDNA libraries. Full-length cDNAs were then used for expression studies by introducing expression plasmids with heterologous viral promoters into BHK [40] or monkey kidney (COS-1) cell lines [41]. Human factor VIII was secreted into the medium of these transfected cells and shown to be active in a clotting assay. Cloning and characterization of the factor VIII gene and its cDNA had been a long task, but its expression was initially surprisingly straightforward.

Development of recombinant factor VIII for clinical use

The subsequent production and purification of recombinant factor VIII from cultured mammalian cells by the biotechnology industry were quite rapid and commensurate with the urgency in producing recombinant factor VIII, which was free of viral contamination for the treatment of haemophilia A patients.

Initially, however, there was some difficulty in the approach developed by Kaufman's group at Genetics Institute in obtaining high yields of factor VIII secreted by CHO cells. Factor VIII cDNA had been introduced into CHO cells by linking it to the selectable, amplifiable marker, dihydrofolate reductase (DHFR). Protein expression levels were, however, much lower than had been observed with other cDNAs, e.g., factor IX, in CHO cells. It emerged that the reason for this was that during intracellular processing of factor VIII in the ER, a significant proportion of the factor VIII became bound to the chaperone, immunoglubulin-binding protein (BiP), through a primary binding site of its A1-domain. Interactions also occurred between factor VIII and 2 other protein chaperones, calnexin and calreticulin, primarily mediated by the B-domain of factor VIII. It was suggested that factor VIII has unique requirements, not shared by the related coagulation factor V, for carbohydrate processing and molecular chaperone interactions that may limit its secretion [42]. In practice the problem of the low yield of secreted factor VIII was initially overcome, at least in part, by the addition of von Willebrand factor (vWF) (the cofactor with which factor VIII is normally associated as a high-molecular-weight complex in plasma) to the tissue culture medium. Thus factor VIII secreted in the absence of vWF appeared to be rapidly degraded. In its presence, however, factor VIII associated with vWF to form high-molecular-weight multimers. This stabilized the factor VIII, protecting it from proteolytic degradation and improving factor VIII yields significantly [43].

Finally, in order to develop a large-scale process for production of factor VIII, the vWF cDNA was introduced into the factor VIII-expressing CHO cells by linking it to a second amplifiable marker, the adenosine deaminase gene. Co-expression of factor VIII and vWF was thereby achieved even in serum-free medium (Fig. 4), improving the factor VIII yields by 1–2 orders of magnitude [44, 45]. Even so, the yield of factor VIII was still significantly lower

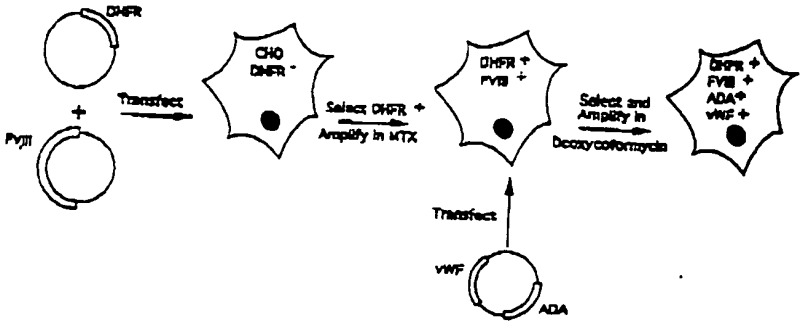

Figure 4. Derivation of factor VIII and von Willebrand factor coexpressing CHO cells used for the manufacture of recombinant factor IX. Adapted from Kaufman [45] with permission. Plasmids encoding DHFR and factor VIII were transfected into DHFR-deficient CHO cells and transformants selected by growth in nucleoside-free medium. Cells were then selected in increasing concentrations of methotrexate to obtain a cell line that had amplified the DHFR and factor VIII genes. These cells were subsequently transfected with a vWF expression plasmid containing an ADA (adenosine deaminase) gene and selected for growth in the presence of cytotoxic concentrations of adenosine with increasing concentrations of 2'-deoxycoformycin. The final cell expressed DHFR, ADA, vWF and factor VIII.

than that of the co-expressed vWF. This suggested that the intracellular regulation of factor VIII mRNA levels and/or regulation of the intracellular folding and processing of factor VIII were still limiting the yields of factor VIII secreted into the culture medium [44]. The factor VIII-expressing CHO cell line is cultured in a defined, serum-free medium in 2500 l bioreactors for the large-scale manufacture of recombinant factor VIII. However, it should be noted that the culture medium is reported to contain (unspecified) added bovine proteins from natural sources, which were, however, tested to minimize the risk of introducing viral contamination [46].

By contrast, a different cell line was used for factor VIII expression in the approach adopted by Genentech/Bayer. A clone of BHK cells was isolated after introducing the factor VIII cDNA, linked to DHFR, into chromosomal DNA followed by amplification of the factor VIII copy number by selection with methotrexate. The BHK clone used for the commercial production of factor VIII contains about 150 copies of the factor VIII cDNA per cell [47]. For the subsequent manufacture of factor VIII in deep-tank stirred suspension fermenters, a proprietary (unspecified), serum-free medium was used [48]. This medium presumably contained some added protein, since there was no requirement to introduce the vWF cDNA to improve the stability of the factor VIII secreted in this BHK cell line, unlike the situation in CHO cells (see above).

Both recombinant factor VIII products (Genetics Institute or Genentech), after purification by immuno-affinity chromatography with monoclonal antibodies, ion-exchange chromatography and other methods, were reported to be essentially indistinguishable from plasma-derived factor VIII [45, 48]. The full-length recombinant factor VIII produced by both companies may be

regarded as a "first-generation" product, since the CHO cells used to produce factor VIII were grown in a tissue culture medium that contained protein additives. Moreover, the resultant purified factor VIII was stabilized by the addition of human albumin. Thus there remains a small theoretical risk, since natural products from humans or cattle were used in their manufacture, that heat-resistant viruses, such as B19 parvovirus (causing fifth disease) or, more seriously, prions, might still be present. Approval was granted in 1992 for the sale, in the USA, of Recombinate (manufactured by Genetics Institute/Baxter) and in 1993 for the sale of Kogenate (manufactured by Genentech/Bayer, originally Miles/Cutter).

B-domainless recombinant factor VIII

A second generation, B-domainless factor VIII, recombinant factor VIII SQ ("Refacto": Pharmacia/Upjohn/Genetics Institute), is now (1999) also approved [49]. Early work, both by recombinant DNA and classic protein chemistry methods, had shown that factor VIII with its B domain deleted was active in clotting assays [50–52]. Factor VIII SQ was developed from one particular construct, which retained the factor VIII processing protease cleavage sites at amino acids 740 and 1649 with only 14 residues of the B domain still present. This was almost fully processed when expressed in CHO cells, consistent with its retaining a furin cleavage site preceding amino acid 1649 [53]. An advantage of factor VIII SQ, in comparison to the first-generation full-length factor VIII products, is that it is formulated without the addition of human albumin. However, human albumin is still added to the tissue culture medium in which the CHO-expression construct is grown [49]. Thus, these second-generation products have a reduced theoretical risk of transmission of human viruses [49].

Clinical experience with recombinant factors

Recombinant products offer the greatest margin of safety for haemophilic patients and have been recommended for the treatment of all patients. However, they are significantly more expensive and many patients continue to receive plasma-derived products. In addition to offering an increased margin of safety with regard to viral contamination, recombinant products also offer a solution to the problem of the burgeoning demand for coagulation factors which continues to rise steeply. Thus 62 million units of factor VIII were used in the UK in 1981, but by 1998 consumption had increased to 200 million units. This largely reflects the changing pattern of treatment, with patients now being encouraged to treat themselves at home on a prophylactic basis to prevent joint bleeds. The plasma half-life and recovery of the various recombinant factor VIII products are identical to those observed with conventional plasma-derived products and are typically around 14–16 hours. In contrast to many plasma-derived products, none of the recombinant products contain vWF, and

thus they are of no use in the treatment of this different congenital haemorrhagic disorder. The plasma half-lives of recombinant factor IX are approximately 18 hours, which are also the same as those observed with plasma-derived factor IX products [37].

Recombinant factor VIIa

Recombinant factor VIIa (Novoseven, Novo Nordisk) has proved very useful in the clinical management of patients with either haemophilia A or B and inhibitory antibodies, as well as those with acquired haemophilia. It by-passes the requirement for either factor VIIIa or IXa because it activates factor X (see Fig. 1). Control of bleeding episodes in patients with inhibitory antibodies is a major clinical challenge. Most of the clinical experience relating to use of recombinant factor VIIa has been gained outside the setting of formal clinical trials, and this has hindered licensing in the United States [54]. The product is extremely expensive and this is likely to hinder widespread use when other products may be just as effective. It is valuable in patients who have high titres of inhibitory antibodies. It is of particular value, however, in the few patients with haemophilia B and inhibitory antibodies, as administration of either plasma-derived or recombinant factor IX in these cases can result in serious anaphylactic reactions [55]. Factor VIIa has a plasma half-life of only approximately two hours, so that frequent bolus injections are required to control bleeding; it is not licensed for continuous intravenous administration. No specific adverse effects have been identified. The excellent safety profile, including lack of allergic reactions, has encouraged some clinicians to employ this product for first-line treatment of bleeds at home [56]. In the past, physicians were often reluctant to undertake elective surgery in haemophiliacs with inhibitors, but such procedures can now be carried out with confidence with factor VIIa [57].

Safety issues

Concerns about recombinant coagulation factor concentrates have focused on three main issues: viral safety, incidence of inhibitor development and problems with laboratory assays. It is somewhat ironic that several currently licensed preparations of recombinant factor VIII actually contain a considerable quantity of human, plasma-derived albumin that is added to the product as a stabilizer. Furthermore, most recombinant products are not subjected to specific virucidal treatment, such as heat treatment, during manufacture. Alternative stabilizers have been developed and clinical trials with these second-generation recombinant factor VIII products are already underway. Recombinant factor IX does not require the addition of albumin as a stabilizer. Bovine proteins are incorporated in growth media used in the manufacture

Table 2. Recombinant factor concentrates

Product and manufacturer	Viral inactivation	Human albumin	Bovine protein
Recombinate (Baxter)	No	Yes	Yes
Kogenate[1] (Bayer)	Yes	No[2]	No
Refacto (Wyeth)	Yes	No[2]	No
BeneFIX (Baxter)	Yes	No	No
Novo Seven (Novo)	No	No	Yes

Footnotes: [1] identical to Helixate (Aventis); [2] although cells are grown in human albumin

of some products (see above). The current status of the various recombinant products is summarized in Table 2.

Concern has also been expressed that the use of highly purified factors might result in a greater incidence of inhibitory antibodies. Unfortunately, it is not possible to give a definitive answer to this important issue, as no prospective, double-blinded clinical studies have been conducted in which the incidence of inhibitor development has been compared in patients receiving recombinant products and those receiving conventional plasma-derived products. Approximately 5% of patients with haemophilia A in the UK are known to have developed inhibitory antibodies (or about 15% if severe patients with a baseline of <2% factor VIII are considered). The first two clinical trials of two different recombinant products in previously untreated patients (PUPs) reported an incidence of inhibitor development of around 20% [58, 59]. Inhibitors appeared after a mean of 9 exposure days in both clinical trials. A more recent study from France with a longer follow-up period reported an inhibitor incidence of 28% in patients receiving recombinant factor VIII, in contrast to an incidence of 9% in a historical control group which received only plasma-derived products [60]. Similar results have been observed in other groups. Most of the cases involved low titre and/or transient inhibitors that disappeared as treatment was continued. It is quite probable that many cases of transient or low-titre inhibitors amongst patients receiving conventional concentrates were simply not identified in the past, and most haematologists do not believe that the incidence of significant inhibitors in patients receiving recombinant products is truly elevated. The median number of exposure days until detection of the inhibitor was 9.5 days (range 5–14 days).

By contrast with recombinant factor VIII, there is no evidence of an increased risk of inhibitor development with the use of recombinant factor IX. In clinical trials, only one of 31 PUPs developed an inhibitor and only one of 56 previously treated patients (PTPs) developed a transient inhibitor. These findings are comparable with the incidence of inhibitors of approximately 1% of historical controls with haemophilia B. The formation of specific antibodies against recombinant factor VIIa has not been identified in subjects with either congenital or acquired haemophilia.

The ability to assay the activity of factor VIII both in actual coagulation factor concentrates and in the plasma of haemophiliac patients after infusion of products is vital for clinical care. Various methods have been developed for the assay of factor VIII, including the one-stage, two-stage and automated chromogenic methods. In general, clinical laboratories tend to prefer the one-stage method, which is both faster and easier to perform, for assay of factor VIII in both concentrates and patient plasma. By contrast, the potency of concentrates is now assigned by the chromogenic method, which is actually based on the two-stage method. Traditionally, human plasma is used as the standard for assays of plasma factor VIII. When plasma levels of factor VIII are assayed after infusion of recombinant factor VIII, recovery is significantly greater when using the chromogenic assay compared to the conventional one-stage assay. Pharmacokinetic evaluation also shows a 25% greater AUC (Area under curve (concentration × time) with the chromogenic method, but half-lives are identical on calculations using either assay method. It has recently been shown that these differences may be resolved by using recombinant concentrate diluted in haemophilic plasma as the laboratory standard. The recovery of recombinant factor IX in patients is slightly lower (on average by about 20%) than that observed with standard, plasma-derived products (see above). This means that a higher dose has to be given to patients to achieve a defined target plasma-level. Marked differences in laboratory assay results have been observed with "Refacto" (B-domainless factor VIII). The potency of this product should be assayed using the chromogenic substrate method, since conventional one-stage assays usually underestimate the real potency.

Summary and prospects

Recombinant factors VIII, IX and VIIa are now available for treatment of haemophilia A & B patients in countries where this newer form of "protein therapy" is affordable. They are safer than plasma-derived factors because they carry no, or minimal, risk of contamination by viruses. Unfortunately, at present, they are more expensive. In the future, gene therapy for haemophiliacs may become available. This subject has certainly been widely discussed as a potential cure for patients and there is considerable current interest in this topic [61]. Good progress has been made with adenoviral and adeno-associated viral vectors and clinical trials are in progress with adeno-associated viral vectors. Thus gene therapy is some way off and may not become available for haemophiliacs for some time.

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References

1 Gailani D, Broze GJ (1991) Factor IX activation in a revised model of blood coagulation. *Science* 253: 909–912
2 Larsson SA (1985) Life expectancy of Swedish haemophiliacs 1831–1980. *Brit J Haematol* 59: 593–602
3 Giannelli F, Choo KH, Rees DJG, Boyd Y, Rizza CR, Brownlee GG (1983) Gene deletions in patients with haemophilia B and anti-factor IX antibodies. *Nature* 303: 181–182
4 Schwaab R, Brackmann HH, Meyer C, Seehafer J, Kirchgesser M, Haack A, Olek K, Tuddenham EGD, Oldenburg J (1995) Haemophilia A: mutation type determines risk of inhibitor formation. *Thromb Haemost* 74: 1402–1406
5 Antonarakis SE, Rossiter JP, Young M, Horst J, de Moerloose P, Sommer SS, Kazazian HH, Negrier C, Vinciguerra C et al (1995) Factor VIII gene inversions in severe hemophilia A: results of an international consortium study. *Blood* 86: 2206–2212
6 Green PM, Giannelli F, Sommer SS, Poon M-C, Ludwig M, Schwaab R, Reitsma PH, Goossens M, Yoshioka A, Figueiredo MS et al (2000) Haemophilia B: database of point mutations and short additions and deletions-v9.0, www.umds.ac.uk/molgen/haemBdatabase.html
7 Naylor JA, Green PM, Rizza CR, Giannelli F (1993) Analysis of factor VIII messenger RNA reveals defects in every one of 28 haemophilia A patients. *Hum Mol Genet* 2: 11–17
8 Rowley G, Saad S, Giannelli F, Green PM (1995) Ultrarapid mutation detection by multiplex, solid-phase chemical cleavage. *Genomics* 30: 574–582
9 Tuddenham EGD (1994) Flip tip inversion and haemophilia A. *Lancet* 342: 307–308
10 Kemball-Cook G, Tuddenham EGD, Wacey AI (1999) Haemophilia A mutation database. http://europium.mrc.rpms.ac.uk
11 Giannelli F, Green PM, Sommer SS, Poon M-C, Ludwig M, Schwaab R, Reitsma PH, Goossens M, Yoshioka A, Figueiredo MS et al (1998) Haemophilia B: database of point mutations and short additions and deletions: eighth edition. *Nucl Acid Res* 26: 265–268
12 Taylor SAM, Duffin J, Cameron C, Teitel J, Garvey B, Lillicrap DP (1992) Characterization of the original Christmas disease mutation (cysteine 206 → serine): from clinical recognition to molecular pathogenesis. *Thromb Haemost* 67: 63–65
13 Morgan GE, Rowley G, Green PM, Chisholm M, Giannelli F, Brownlee GG (1997) Further evidence for the importance of an androgen response element in the factor IX promoter. *Brit J Haematol* 98: 79–85
14 Biggs R (1967) Thirty years of haemophilia treatment in Oxford. *Brit J Haematol* 13: 452–463
15 Rosendaal FR, Varekamp I, Smit C, Brocker-Vriends AHJT, van Dijck H, Vandenbroucke JP, Hermans J, Suurmeijer TPBM, Briet E (1989) Mortality and causes of death in Dutch haemophiliacs 1972–86. *Brit J Haematol* 71: 71–76
16 Darby SC, Ewart DW, Giangrande PLF, Dolin PJ, Spooner RJD, Rizza CR on behalf of the UKHaemophilia Centre Directors' Organization (1995) Mortality before and after HIV infection in the complete UK population of haemophiliacs. *Nature* 377: 79–82
17 Darby SC, Ewart DW, Giangrande PLF, Spooner RJD, Rizza CR, Dusheiko GM, Lee CA, Ludlam CA, Preston FE, for the UK Haemophilia Centre Directors' Organization (1997) Mortality from liver cancer and liver disease in haemophilic men and boys in UK given blood products contaminated with hepatitis C. *Lancet* 350: 1425–1431
18 Lee CA, Ironside JW, Bell JE, Giangrande PLF, Ludlam CA, Estiri MM, McLaughlin JE (1998) Retrospective neuropathological review of prion disease in UK haemophilic patients. *Thromb Haemost* 80: 909–911
19 Kamiyama K, Ericsson LH, Enfield DL, Walsh KA, Neurath H, Davie EW, Titani K (1979) Comparison of amino acid sequence of bovine coagulation factor IX (Christmas factor) with that of other vitamin K-dependent plasma proteins. *Proc Natl Acad Sci USA* 76: 4990–4994
20 Proudfoot NJ, Brownlee GG (1974) Sequence at the 3' end of globin mRNA shows homology with immunoglobulin light chain mRNA. *Nature* 252: 359–362
21 Winter G, Fields S, Gait MJ, Brownlee GG (1981) The use of synthetic oligodeoxynucleotide primers in cloning and sequencing segment 8 of influenza virus A/PR/8/34. *Nucl Acid Res* 9: 237–245
22 Fields S, Winter G, Brownlee GG (1981) Structure of the neuraminidase gene in human influenza virus A/PR/8/34. *Nature* 290: 213–217
23 Choo KH, Gould KG, Rees DJG, Brownlee GG (1982) Molecular cloning of the gene for human antihaemophilic factor IX. *Nature* 299: 178–180

24 Wallace RB, Johnson MJ, Hirose T, Miyake T, Kawashima EH, Itakura K (1981) The use of synthetic oligonucleotides as hybridization probes: II. Hybridization of oligonucleotides of mixed sequence to rabbit β-globin DNA. *Nucl Acid Res* 9: 879–898

25 Kurachi K, Davie EW (1982) Isolation and characterization of a cDNA coding for human factor IX. *Proc Natl Acad Sci USA* 79: 6461–6464

26 Jaye M, de la Salle H, Schamber F, Balland A, Kohli V, Findeli A, Tolstoshev P, Lecocq J-P (1983) Isolation of a human anti-haemophilic factor IX cDNA clone using a unique 52-base synthetic oligonucleotide probe derived from the amino acid sequence of bovine factor IX. *Nucl Acid Res* 11: 2325–2335

27 Anson DS, Choo KH, Rees DJG, Gianneli F, Gould K, Huddleston JA, Brownlee GG (1984) Gene structure of human anti-haemophilic factor IX. *EMBO J* 3: 1053–1064

28 Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K (1985) Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry* 24: 3736–3750

29 Bentley AK, Rees DJG, Rizza C, Brownlee GG (1986) Defective propeptide processing of blood clotting factor IX caused by mutation of arginine to glutamine at position-4. *Cell* 45: 343–348

30 Anson DS, Austen DEG, Brownlee GG (1985) Expression of active human clotting factor IX from recombinant DNA clones in mammalian cells. *Nature* 315: 683–685

31 Anson DS, Brownlee GG, Jones IM (1992) Factor IX preparations uncontaminated by plasma components or poxvirus. *US patent no.* 5,171,569

32 Busby S, Kumar A, Joseph M, Halfpap L, Insley M, Berkner K, Kurachi K, Woodbury R (1985) Expression of active human factor IX in transfected cells. *Nature* 316: 271–273

33 de la Salle H, Altenburger W, Elkaim R, Dott K, Dieterle A, Drillien R, Cazenave J-P, Tolstoshev P, Lecocq J-P (1985) Active γ-carboxylated human factor IX expressed using recombinant DNA techniques. *Nature* 316: 268–270

34 Kaufman RJ, Wasley LC, Furie BC, Furie B, Shoemaker CB (1986) Expression, purification and characterization of recombinant γ-carboxylated factor IX synthesized in chinese hamster ovary cells. *J Biol Chem* 261: 9622–9628

35 Rehemtulla A, Roth DA, Wasley LC, Kulliopulos A, Walsh CT, Furie B, Furie BC, Kaufman RJ (1993) *In vitro* and *in vivo* functional characterization of bovine vitamin K-dependent γ-carboxylase expressed in chinese hamster ovary cells. *Proc Natl Acad Sci USA* 90: 4611–4615

36 Wasley LC, Rehemtulla A, Bristol JA, Kaufman RJ (1993) PACE/furin can process the vitamin K-dependent pro-factor IX precursor within the secretory pathway. *J Biol Chem* 268: 8458–8465

37 White GC, Beebe A, Nielsen B (1997) Recombinant factor IX. *Thromb Haemost* 78: 261–265

38 Gillis S, Furie BC, Furie B, Patel H, Huberty MC, Switzer M, Foster WB, Scoble HA, Bond MD (1997) γ-Carboxyglutamic acids 36 and 40 do not contribute to human factor IX function. *Protein Sci* 6: 185–196

39 Brownlee GG, Rizza C (1984) Clotting factor VIII cloned. *Nature* 312: 307

40 Wood WI, Capon DJ, Simonsen CC, Eaton DL, Gitschier J, Keyt B, Seeburg PH, Smith DH, Hollingshead P, Wion KL et al (1984) Expression of active human factor VIII from recombinant DNA clones. *Nature* 312: 330–337

41 Toole JJ, Knopf JL, Wozney JM, Sultzman LA, Buecker JL, Pittman DD, Kaufman RJ, Brown E, Shoemaker C, Orr EC et al (1984) Molecular cloning of a cDNA encoding human antihaemophilic factor. *Nature* 312: 342–347

42 Pipe SW, Morris JA, Shah J, Kaufman R (1998) Differential interaction of coagulation factor VIII and factor V with protein chaperones calnexin and calreticulin. *Proc Natl Acad Sci USA* 273: 8537–8544

43 Kaufman RJ, Wasley LC, Dorner AJ (1988) Synthesis, processing and secretion of recombinant factor VIII expressed in mammalian cells. *J Biol Chem* 263: 6352–6362

44 Kaufman RJ, Wasley LC, Davies MV, Wise RJ, Israel DI, Dorner AJ (1989) Effect of von Willebrand factor coexpression on the synthesis and secretion of factor VIII in chinese hamster ovary cells. *Mol Cell Biol* 9: 1233–1242

45 Kaufman RJ (1992) Expression and structure-function properties of recombinant factor VIII. *Transfus Med Rev* 6: 235–246

46 Gomperts E, Lundblad R, Adamson R (1992) The manufacturing process of recombinant factor VIII, Recombinate. *Transfus Med Rev* 6: 247–251

47 Chan SY, Lembach KJ (1991) Genetic characterization of recombinant BHK-21 cells expressing factor VIII. *Semin Hematol* 28: 10–16

48 Klein U (1991) Production and characterization of recombinant factor VIII. *Semin Hematol* 28: 17–21

49 Berntop E (1997) Second generation, B-domain deleted recombinant factor VIII. *Thromb Haemost* 78: 256–265

50 Toole JJ, Pittman DD, Orr EC, Murtha P, Wasley LC, Kaufman R (1986) A large region (≈ 95 kDa) of human factor VIII is dispensable for *in vitro* procoagulant activity. *Proc Natl Acad Sci USA* 83: 5939–5942

51 Andersson L-O, Forsman N, Huang K, Larsen K, Lundin A, Pavlu B, Sandberg H, Severin K, Smart J (1986) Isolation and characterization of human factor VIII: Molecular forms in commercial factor VIII concentrate, cryoprecipitate, and plasma. *Proc Natl Acad Sci USA* 83: 2979–2983

52 Eaton DL, Wood WI, Eaton D, Hass PE, Hollingshead P, Wion K, Mather J, Lawn RM, Vehar GA, Gorman C (1986) Construction and characterization of an active factor VIII variant lacking the central one-third of the molecule. *Biochemistry* 25: 8343–8347

53 Lind P, Larsson K, Spira J, Sydow-Backman M, Almstedt A, Gray E, Sandberg H (1995) Novel forms of B-domain-deleted recombinant factor VIII molecules. Construction and biochemical characterization. *Eur J Biochem* 232: 19–27

54 Lusher J, Ingerslev J, Roberts H, Hedner U (1998) Clinical experience with recombinant factor VIIa. *Blood Coagulat Fibrinolys* 9: 119–128

55 Warrier I, Ewenstein BM, Koerper MA, Shapiro A, Key N, Dimichele D, Miller RT, Pasi J, Rivard GE, Sommer SS et al (1996) Factor IX inhibitors and anaphylaxis in haemophilia B. *Haemophilia* 2: 239–260

56 Key NS, Aledort LM, Beardsley D, Cooper HA, Davignon G, Ewenstein BM, Gilchrist GS, Gill JC, Glader B, Hoots WK et al (1998) Home treatment of mild to moderate bleeding episodes using recombinant factor VIIa (Novoseven) in haemophiliacs with inhibitors. *Thromb Haemost* 80: 912–918

57 Shapiro AD, Gilchrist GS, Hoots WK, Cooper HA, Gastineau DA (1998) Prospective, randomised trial of two doses of rVIIa (NovoSeven) in haemophilia patients with inhibitors undergoing surgery. *Thromb Haemost* 80: 773–778

58 Lusher JM, Arkin S, Abildgaard CF, Schwartz RS (1993) Recombinant factor VIII for the treatment of previously untreated patients with haemophilia. *N Engl J Med* 328: 453–459

59 Bray GL, Gomperts ED, Courter S, Gordon EM, Manco-Johnson M, Shapiro A, Scheibel E, White G, Lee M (1994) A multicenter study of recombinant factor VIII (Recombinate): safety, efficacy, and inhibitor risk in previously untreated patients with haemophilia. *Blood* 83: 2428–2435

60 Rothschild C, Lamrisn Y, Saint-Remy JM, Borel-Derlon A, Chambost H, Morean P, Goudemand J (1998) French previously untreated patients with severe haemophilia A after exposure to recombinant factor VIII: incidence of inhibitor and evaluation of immune tolerance. *Thromb Haemost* 80: 779–783

EXHIBIT C

TRANSFUSION

BLOOD COMPONENTS REFERENCE MANUAL

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Revised: November 1999

Descripti ns, Indications, and Therapeutic Effects
Factor Concentrates

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Factor VIII Concentrates

Description

Factor VIII concentrates are a commercially prepared, lyophilized powder purified from human plasma to treat patients with hemophilia A or von Willebrand's disease. Alternatively, recombinant (synthetic) protein is purified from genetically engineered non-human cells grown in tissue culture. The quantity of factor VIII coagulant activity is stated on the bottle. One factor VIII concentrate unit equals the clotting activity in 1 ml of fresh plasma. Factor VIII concentrate is cell free and is administered without regard to patient or donor ABO or Rh type. It is heat treated and/or solvent detergent treated to reduce the risk of virus transmission. Current processes appear to have eliminated the risk of HIV, HBV and HCV transmission. Concentrates differ in the purification procedures. Highly purified factor VIII, e.g. preparations purified over a monoclonal antibody column or current recombinant factor VIII concentrates, are stabilized by adding 98% of pasteurized human albumin. Porcine factor VIII concentrate is available for patients with high titer anti-human factor VIII allo or autoantibody inhibitors. Factor VIII concentrates are stored refrigerated at 2-8°C for up to two years from the date of manufacture (expiration date will be indicated on each vial). Some preparations may be kept at room temperature for extended periods. Once reconstituted, it should not be refrigerated. Factor VIII concentrate should be infused within 4 hours of preparation to reduce the risk of bacterial growth. Vials are usually shipped to a hospital pharmacy, blood service or nursing unit and mixed there prior to use. Many patients or families receive them directly for home care.

Indication

Factor VIII concentrate is indicated for the treatment of bleeding or imminent invasive procedures in patients with hemophilia A (congenital factor VIII deficiency) and for patients with low titer factor VIII inhibitors. Regular prophylactic doses are sometimes used as well as daily doses in some hemophilic inhibitor patients to try to induce immune tolerance. Patients with von Willebrand's disease respond to one specific, pasteurized intermediate-purity concentrate in which von Willebrand factor activity is relatively preserved.

Therapeutic Effect

Dosage is dependent on the nature of the injury, the degree of factor deficiency, the weight of the patient and the presence and level or absence of factor VIII inhibitors. The half life of circulating factor VIII is 8 to 12 hours, therefore transfusions may need to be repeated every 12 to 24 hours to maintain hemostatic levels. Following surgery, it is necessary to maintain hemostatic levels for up to two weeks to prevent delayed bleeding and promote wound healing in the hemophilic patient. The Puget Sound Blood Center's Hemophilia Program is available for consultation at 206-292-6507 (or 1-800-552-0640) or 206-292-6525 (and page) on evenings or weekends.

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EXHIBIT D

Emory Health Sciences Press Release

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Oct ber 10, 2000

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EMORY'S COMPREHENSIVE HEMOPHILIA PROGRAM IS A LEADER IN CLINICAL RESEARCH AND ADVANCED TREATMENTS FOR ADULTS AND CHILDREN

The comprehensive hemophilia program at Emory University has become a national leader in research and treatment programs that are helping improve the lives of the 15,000 hemophiliacs living in the U.S. Although these patients now can enjoy a normal life span, they must rely on infusions of blood products to treat frequent bleeding episodes, then cope with resulting complications, including inhibitors that render the products ineffective and the threat of infections and joint diseases caused by internal bleeding. In the past, the cure for hemophilia has sometimes been worse than the disease.

Emory hematologists participate in a number of clinical trials to improve treatments for hemophilia patients, including a recently concluded international study sponsored by Bayer to test a new formulation of blood clotting products. The new formula, called Kogenate FS, is almost completely free of any human or animal components and should make hemophiliacs feel safer than ever about using genetically engineered clotting factors. Kogenate FS has recently been approved by the Food and Drug Administration.

Individuals with hemophilia are missing the gene that makes Factor VIII, a critical part of the blood clotting machinery. Factor VIII is a component of fibrin, which is the cement the body uses to seal a wound after platelets first plug it up, much like the Dutch boy putting his finger in the dike while awaiting help.

In the past, Factor VIII products to treat hemophilia patients were made by concentrating clotting factor gathered from the plasma of a large group of donors. In the mid 1980s, when scientists discovered that these blood products could transmit diseases like HIV and hepatitis C, they began heating Factor VIII products to kill these viruses.

In the early 1990s, scientists carried safety one step further with genetically engineered recombinant Factor VIII products made by inserting the factor VIII gene into a cell line and producing mass quantities of concentrated human factor VIII. Although these products contained no human or animal products, they were stabilized with a small amount of albumin, a human blood component. Kogenate FS -- the newest FDA-approved product -- uses small amounts of albumin in the initial "fermenting" solution, but in the final stage, albumin is removed, leaving the product almost completely free of any human or animal components. The Factor VIII is then stabilized with sucrose. The product is said to be sucrose-formulated and albumin-free in final formulation.

"Our research found that the new product works just as well as the current products and appears to offer a greater safety margin against infectious agents," says Thomas Abshire, M.D., medical director of Emory's hemophilia program and one of the principal investigators for the study.

Emory has just completed another randomized study, in collaboration with Schering Plough, Inc. and the American Red Cross, in which hemophilia patients with hepatitis C were treated either with a combination of interferon and Ribavirin, or with interferon alone, which is the

known treatment. Preliminary results presented at the World Federation of Hemophilia in July determined that the combination therapy is better than interferon alone.

Emory also is a world leader in treating joint disease in hemophiliacs -- a common problem caused by bleeding into joints, which causes irritation in the lining of the joint cavity and creates a cycle of bleeding and inflammation. Surgeons and hematologists have collaborated on a study of arthroscopic synovectomy, in which a small endoscope is inserted into the ankle, elbow, or knee to clean out the thickened lining. When surgery is not an option, physicians use an alternative technique called radionuclide synovectomy to inject a radioisotope into the joint that eliminates the abnormal lining. Drs. Michael Busch and Amy Dunn coordinate this program.

Emory's comprehensive adult and pediatric hemophilia program includes hematologists, infectious disease specialists, hepatologists, orthopaedic surgeons, physical therapists and specialty nurses. The program receives some federal funding through the Maternal and Child Health Bureau (MCHB) and the Centers for Disease Control and Prevention (CDC). Two adult and two pediatric hematologists treat 350 patients, including 140 children. The staff also works closely with a program at Children's Healthcare of Atlanta at Scottish Rite, which treats 150 additional children. Dr. Abshire also is Medical Director for MCHB Region IV South hemophilia programs that include Alabama, Mississippi, Georgia and Florida.

Although the number of hemophilia patients is small relative to many other diseases, it commands a great deal of attention because it is so expensive to treat. Clotting factor for a mild joint bleed in a typical 7-year-old child, even at reduced rates, averages $600 per infusion, with some patients needing several infusions per week. "There is a motivation to produce a better product and one you can use less of, which may come with the eventual development of gene therapy for Factor VIII," says Dr. Abshire.

Other current clinical trials at Emory aimed at treating bleeding disorders and their complications include: (1) a study of children who experience clotting problems from permanent IVs; (2) a CDC-sponsored study designed to identify and treat women with undiagnosed bleeding disorders who are experiencing abnormal bleeding with menstrual periods (hematologist Sidney Stein, M.D., leads the Emory component of this multi-site study); (3) multiple clinical trials designed to evaluate the safety and efficacy of new products used to treat bleeding episodes; (4) multiple AIDS Clinical Trials Group (ACTG) studies for our patients that contracted HIV infection from blood products prior to the development of safer products.

The Emory hemophilia treatment center also participates in studies designed to identify the safest and most cost effective methods for preventing the complications of congenital bleeding disorders, including the CDC-sponsored Universal Data Collection System and the National Hemophilia Foundation's National Prevention and Awareness Campaign.

Exciting research advances are on the horizon to deal with the problems faced by the 20 percent of hemophilia patients who have antibodies that inhibit the effectiveness of substitute Factor VIII products. Emory hematologist J.S. "Pete" Lollar, M.D., is conducting groundbreaking research in the laboratory that includes gene therapy and an improved Factor VIII molecule constructed from a combination of human and pig Factor VIII genes.

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For more general information on The Robert W. Woodruff Health Sciences Center
all Health Sciences Communication's Office at 404-727-5686,
or send e-mail to hsnews@emory.edu

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Last Updated: October 13, 2000

EXHIBIT E

News

January 10, 2001

Refacto (rFVIII) to Become Available

Wyeth/Genetics Institute has announced that their second-generation recombinant factor VIII product, ReFacto will be available in the United States beginning on January 29, 2001. ReFacto is the first recombinant factor VIII product formulated without human serum albumin in its final formulation.

ReFacto is indicated for the control and prevention of hemorrhagic episodes and for surgical prophylaxis in patients with hemophilia A. ReFacto is also indicated for short-term routine prophylaxis to reduce the frequency of spontaneous bleeding episodes.

Wyeth/Genetics Institute claims that dosing for ReFacto will be exactly the same as the other second-generation recombinant factor VIII products currently on the market. Further, Wyeth/Genetics Institute has announced that 2000 I.U. sizes for both ReFacto and their recombinant factor IX, BeneFix, will be available.

Due to limited supply of ReFacto however, Wyeth/Genetics Institute will be placing limits on individual customer size orders. Wyeth/Genetics Institute has devised an allocation method whereas the 18 largest homecare companies and the hemophilia treatment centers will be allocated product based on the percentage of hemophilia A patients they see. For example, if ABC Homecare has a hemophilia A base of 10% of the overall hemophilia A population, they would be allocated 10% of Wyeth/Genetic Institute's inventory of ReFacto.

Increased production for ReFacto is expected sometime next year as Wyeth/Genetics Institute is working towards completion of a refurbished manufacturing plant in St. Louis, Missouri. Currently, product is being imported from a manufacturing facility in Stockholm, Sweden. The St. Louis facility is forecast for completion in early 2002.

The list price for ReFacto will be $1.09. However, Wyeth/Genetics Institute is going to offer a bulk contract price of $0.84. The list price for other second-generation recombinant factor VIII products are similar (Kogenate FS - $1.13; Helixate FS - $1.11).

The Average Wholesale Price (AWP) of ReFacto is expected to be the similar to other second-generation recombinant factor VIII's as well. Wyeth/Genetics Institute expects the

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AWP of ReFacto to be the list price ($1.09) multiplied by 25%. It this is the case, ReFacto's AWP would be $1.36 whereas Kogenate FS would be $1.41 and Helixate FS $1.38.

Wyeth/Genetics Institute is also working on a third-generation recombinant factor VIII named ReFacto AF (Albumin Free). The St. Louis manufacturing facility has been selected to product R Facto AF, once the FDA approves that product. Wyeth/Genetics Institute has yet to decide if they will phase out ReFacto once ReFacto AF has been approved. In similar situations, Bayer chose to phase out their first generation recombinant product, Kogenate, once their second-generation product, Kogenate FS was licensed. However, Baxter Hyland Immuno has decided to keep their first generation recombinant product Recombinate on the market, even after their third-generation recombinant product is licensed.

Wyeth/Genetics Institute currently manufactures and distributes ReFacto in Europe. Moreover, they are currently the only manufacturers of a recombinant factor IX product, BeneFix.

EXHIBIT F

US006171825B1

(12) **United States Patent**
Chan et al.

(10) **Patent No.: US 6,171,825 B1**
(45) **Date of Patent: Jan. 9, 2001**

(54) **PREPARATION OF RECOMBINANT FACTOR VIII IN A PROTEIN FREE MEDIUM**

(75) Inventors: **Sham-Yuen Chan**, El Sobrante; **Kathleen Harris**, Oakland, both of CA (US)

(73) Assignee: **Bayer Corporation**, Berkeley, CA (US)

(*) Notice: Under 35 U.S.C. 154(b), the term of this patent shall be extended for 0 days.

(21) Appl. No.: **09/146,708**

(22) Filed: **Sep. 4, 1998**

Related U.S. Application Data

(63) Continuation-in-part of application No. 08/844,714, filed on Apr. 18, 1997, now Pat. No. 5,804,420.

(51) **Int. Cl.**[7] **C12P 21/04**; C12N 5/00; A61K 35/14; C07K 14/00

(52) **U.S. Cl.** **435/69.6**; 435/383; 435/404; 435/406; 530/383

(58) **Field of Search** 435/69.6, 172.3, 435/325, 352, 363, 373, 383, 404, 406; 530/383

(56) **References Cited**

FOREIGN PATENT DOCUMENTS

WO98/15614 * 4/1998 (WO) 435/325

OTHER PUBLICATIONS

Gibco BRL Catalogue and Reference Guide, Life Technologies, Inc., 1991.*

* cited by examiner

Primary Examiner—Karen Cochrane Carlson
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(74) *Attorney, Agent, or Firm*—James A. Giblin

(57) **ABSTRACT**

Recombinant Factor VIII can be produced in relatively large quantities on a continuous basis from mammalian cells in the absence of any animal-derived proteins such as albumin by culturing the cells in a protein free medium supplemented with polyol copolymers, preferably in the presence of trace metals such as copper. In very preferred embodiments, the medium includes a polyglycol known as Pluronic F-68, copper sulfate, ferrous sulfate/EDTA complex, and salts of trace metals such as manganese, molybdenum, silicon, lithium and chromium. With an alternative medium which included trace copper ions alone (without polyol copolymers) we were also able to enhance the productivity of Factor VIII in recombinant cells such as BHK cells that are genetically engineered to express Factor VIII.

11 Claims, No Drawings

PREPARATION OF RECOMBINANT FACTOR VIII IN A PROTEIN FREE MEDIUM

This is a continuation-in-part of prior application Ser. No. 08/844,714 filed Apr. 18, 1997 now U.S. Pat. No. 5,804,420.

BACKGROUND OF THE INVENTION

1. Field

This disclosure is concerned generally with the manufacture of recombinant Factor VIII and specifically with the manufacture of recombinant Factor VIII in a serum or protein free medium.

2. Prior Art

Hemophilia A is an X-linked recessive genetic disorder that is due to a defective or deficient Factor VIII molecule, resulting in a hemorrhagic tendency. To control bleeding episodes, hemophiliacs are treated with Factor VIII. Historically Factor VIII has been isolated from human blood plasma. However, therapy with plasma-derived Factor VIII has been associated with transmission of several human viruses, such as hepatitis and human immunodeficiency viruses.

With the advent of recombinant DNA technology, the structure of human Factor VIII and its gene has been elucidated. The transcription product of the gene, which is derived from 26 exons, is a messenger RNA molecule of ~9000 bases in length, coding for a large protein of 2351 amino acids. Structural studies of Factor VIII indicate that it is a glycoprotein containing a significant number of carbohydrate residues.

The cDNA coding for Factor VIII has been cloned and stably expressed in baby hamster kidney (BHK-21) and Chinese hamster ovary (CHO) cells. Commercial processes have been developed to produce recombinant Factor VIII for treatment of hemophilia A.

Recombinant Factor VIII is currently manufactured by genetically engineered mammalian cells, thus obviating the reliance on plasma and minimizing any possible risk of virus transmission.

Gene amplification has been the method of choice to derive high production cell lines for therapeutic proteins. The amplification strategy involves the linking of a transcriptional unit encoding the desired protein to an amplifiable marker such as dihydrofolate reductase. Transfection techniques are then applied to transfer the vector DNA to recipient cells. Cell populations are selected for increased resistance to the drug of choice such as methotrexate. The establishment of a stable cell clone is accomplished by limiting dilution cloning. These cell clones are then adapted to a serum-free production medium and monitored for production of the desired protein.

For labile proteins such as Factor VIII, human albumin has been added as a stabilizer during the preparation and purification procedures. Although the albumin is subjected to a viral inactivation step by pasteurization, it would be ideal if recombinant Factor VIII could be manufactured in the complete absence of human and animal blood proteins. I have now found this is possible by using novel cell culture media. Details are described below.

SUMMARY OF INVENTION

The method for the continuous production of relatively large quantities of recombinant Factor VIII (rFVIII) from mammalian cells in the absence of any human or animal-derived plasma proteins comprises culturing the mammalian host cells in a protein-free medium supplemented with a polyol polymer such as Pluronic F-68 and copper ions. The preferred medium includes copper sulfate, a ferrous sulfate/EDTA complex, and the salts of trace metals such as manganese, molybdenum, silicon, lithium, and chromium. Alternatively we have also found that addition of copper ions alone (without polyol polymers) in the protein free medium may be used to enhance productivity of rFVIII in recombinant mammalian host cells, as described below under Additional Studies.

DETAILED DESCRIPTION OF THE INVENTION

Recent advances in recombinant protein expression technology have made possible the production of protein in large quantities in mammalian cells. Host cells suitable for Factor VIII production include cell lines such as baby hamster kidney (BHK) cells, Chinese hamster ovary (CHO) cells, and human embryonic kidney (HEK) cells. Particularly preferred are baby hamster kidney cells, specifically those transfected with a gene capable of directing the expression of Factor VIII as described in Wood et al. (1984) (including derivatives such as clonal variants and progeny thereof). Such a cell line has been deposited with the American Type Culture Collection and has been assigned the accession number ATCC CRL-8544.

The desired host cell line carrying the Factor VIII gene is typically adapted to grow as suspension cultures in a protein-free production medium which is supplemented with lipoprotein. The basal medium chosen for culturing the host cell line is not critical to the present invention and may be any one of, or combination of those known to the art which are suitable for culturing mammalian cells. Media such as Dulbecco's Modified Eagle Medium, Ham's Medium F-12, Eagle's Minimal Essential Medium, and RPMI-1640 Medium, and the like, are commercially available. The addition of growth factors such as recombinant insulin is conventional in the art.

Due to the labile nature of Factor VIII, the productivity of the engineered host cells is severely reduced under protein-free conditions. Human serum albumin is commonly used as a serum-free culture supplement for the production of recombinant proteins. Human serum albumin serves many functions including: (1) as a carrier for fatty acids, cholesterol and lipophilic vitamins, steroid hormones and growth factors; (2) as a protective agent against damages due to shear forces; (3) as a buffer for pH changes; and (4) as an osmotic pressure regulator. Another critical role of albumin is perhaps to protect labile proteins such as Factor VIII from proteolysis by serving as a substrate for proteases.

The impurities present in albumin preparations may also contribute to the stabilizing effect of albumin. Factors such as lipoprotein (Chan, 1996) have been identified as a replacement for human serum albumin for the production of recombinant Factor VIII under serum-free conditions.

Our attempt to develop a production medium free of human plasma-derived albumin led to the inventions of this disclosure, basal protein-free media for recombinant Factor VIII production. The preferred medium consists of modified Dulbecco's Minimum Essential Medium and Ham's F-12 Medium (50:50, by weight) supplemented with recombinant insulin (Nucellin, Eli Lilly) at 10 μg/ml, and $FeSO_4$•EDTA (50 μM). With the exception of Factor VIII production, engineered BHK cells grow well in this protein-free basal medium.

Surprisingly, the addition of a polyol such as Pluronic F-68 had no effect on growth but enhanced the specific productivity of the BHK cells for Factor VIII. Serendipitously, the addition of copper sulfate further enhances the production of Factor VIII. Also the inclusion of a panel of trace metals such as manganese, molybdenum, silicon, lithium, and chromium lead to further increases in Factor VIII production. A continuous process was then developed for Factor VIII production under human plasma-derived protein-free conditions. Further information regarding the use of Pluronic polyols can be found in Papoutsakis (1991) and Schmolka (1977).

Pluronic F-68, a polyglycol, (BASF, Wyandot) is commonly used to prevent foaming that occurs in stirred cultures, and to protect cells from shear stress and bubble damage in sparged cultures. Pluronic F-68 is a nonionic block copolymer with an average molecular weight of 8400, consisting of a center block of poly(oxypropylene) (20% by weight) and blocks of poly(oxyethylene) at both ends. Extensive research on the role of Pluronic F-68 indicates that Pluronic F-68 acts as a surfactant and prevents damage to cells by allowing the drainage of cells away from bubbles formed in the bioreactors during stirring or sparging. However, several investigators have noticed beneficial effects of Pluronic F-68 on growth under culture conditions in which shear is minimal (Mizrahi, 1975; Murhammer and Goochee, 1990). Co-purification of lipids with Pluronic F-68 during product purification provides anecdotal evidence that the Pluronic polymer may substitute for albumin not only as a surfactant, but may also act as a carrier for lipids. Pluronic F-68 may also prevent membrane damage from killing cells before repair can be effected, possibly by direct intercalation into the membrane. The role of Pluronic F-68 in acting as a metal ions buffer is completely unknown.

Although there are reports that Pluronic F-68 in media can increase volumetric productivity, the mechanism of action appears to be maintenance of cell viability (Schneider, 1989; Qi, 1996). To our knowledge, this is the first time that Pluronic F-68 has been seen to increase specific production of a particular protein product. Since viabilities and growth rates are comparable in our system with and without Pluronic F-68, maintenance of cell viability cannot be the mechanism of action of Pluronic F-68 in our system. However, the effect of Pluronic F-68 addition is immediate and dramatic, whatever the mechanism.

It is anticipated that a range of other polyols would have similar effects. Such other polyols include nonionic block copolymers of poly(oxyethylene) and poly(oxypropylene) having molecular weights ranging from about 1000 to about 16,000.

In addition to conventional suspension culturing techniques such as shake flasks, spinner flasks, and roller bottles, the method of the present invention is also applicable for use with perfusion and batch bioreactors. Following culturing the host cells, the Factor VIII may be recovered from the spent medium by standard methodologies such as ultrafiltration or centrifugation. If desired, the recovered Factor VIII may be purified by, for example, ion exchange or size exclusion chromatography, immuno-affinity or metal chelate chromatography, and the like.

As used herein, a "human or animal protein-free medium" is a cell culture medium which is free of any protein that has been derived from a human source or an animal source. Proteins which are isolated from human or animal sources (such as plasma) inherently carry the risk of introducing viral contamination. The goal of a human or animal protein-free medium is thus to eliminate or at least greatly reduce the risk of viral transmission.

EXAMPLE 1

Baby hamster kidney (BHK-21) cells transfected with a gene capable of directing the expression of Factor VIII were obtained from Genentech, Inc., South San Francisco, Calif., U.S.A. The cell line was prepared as described in detail in Wood et al. (1984) and was deposited with the American Type Culture Collection and given accession number ATCC CRL-8544. A clonal variant of this cell line was also obtained from Genentech, Inc., and used in all examples.

The BHK-21 cells containing the gene encoding Factor VIII were cultivated as suspension cultures in shake flasks using a serum-free basal medium containing the following: Ham's F-12 Medium and Dulbecco's Minimal Essential Medium (50:50, by weight), Nucellin (recombinant insulin, 5–10 μg/ml), $FeSO_4$•EDTA (50 μM), and $MgCl_2$ (15 mM). Cells were maintained and passaged at 48 hour intervals. Cells were spun down at 800xg for 5 minutes, counted and re-seeded at a density of 1×10^6 cells per ml. Each flask contains 50–100 ml of fresh medium. The shake flasks were placed on a rotator, incubated at 37° C., and maintained as suspension culture by swirling gently between 90–110 r.p.m. The effect of a polyol such as Pluronic F-68 (0.1%), shown as F-68 below, and copper sulfate (50 nM) on Factor VIII production was examined in shake flasks. Factor VIII was quantitated by a chromogenic assay. The assay is sold commercially as a test kit known as Coatest VIII:C/4 and is available from Baxter HealthCare Products. The cells were maintained by this procedure for 24 days. The Factor VIII activity in each medium, as determined with the Coatest VIII:C/4 kit, is shown in Table 1.

TABLE 1

Conditions	Titer (U/ml)	Specific Productivity (μU/cell/day)	% Increase over basal
Basal Medium	0.15 ± 0.07*	0.026 ± 0.013	0.0000
Basal + F-68 (0.1%)**	0.24 ± 0.04	0.052 ± 0.013	200
Basal + F-68 (0.1%) + Cu (50 nM**)	0.42 ± 0.09	0.091 ± 0.013	350

*Mean of 36 samples ± standard deviations. The cells were monitored for Factor VIII production over a period of 24 days as described above.
**Titration experiments showed that 0.1% is the optimal dose for Pluronic F-68. Increasing the concentration to 0.3% had no significant impact on Factor VIII production. Dose-response experiments revealed that 50–800 nM copper sulfate is optimal for Factor VIII production.

As shown in Table 1, the addition of Pluronic F-68 alone or, preferably, in combination with copper sulfate significantly enhanced the titer and specific productivity of BHK cells containing the gene encoding Factor VIII under protein-free conditions.

EXAMPLE 2

To further optimize the production of Factor VIII under protein-free conditions, trace metals were added to the protein-free production medium. Factor VIII production was then assessed by the continuous shake flask culture system as described in example 1 for 16 days. The data is shown in Table 2. In the absence of copper sulfate, the trace metals had no effect on Factor VIII productivity. See Table 2.

TABLE 2

Conditions	Titer (U/ml)	Specific Productivity (μU/cell/day)	% Increase over basal + F-68
Basal + F-68	0.46 ± 0.11	0.065 ± 0.013	0.0000
Basal + F-68 + Cu	0.53 ± 0.15	0.078 ± 0.026	120
Basal + F-68 + Cu + metals*	0.73 ± 0.16	0.104 ± 0.026	160

*Metals include $CuSO_4.5H_2O$ (50 nM), $MnSO_4$ (3 nM), $Na_2SiO_3.9H2O$ (1.5 μM), $[NH_4]_6Mo_7O_{24}.4H2O$ (3 nM), $CrK(SO_4)_2.4H_2O$ (1.5 nM), and LiCl (236 nM).

EXAMPLE 3

The effect of trace metals and copper on factor VIII production was further evaluated in a perfusion fermenter. Two 1.5-liter fermenters were seeded with the BHK clonal variant at a density of 2×10^6 cells/ml using the basal medium described in Table 1. The fermenter was perfused at a rate of 0.5 liter/day. One fermenter was kept as a control and the other fermenter was supplemented with copper and trace metals as described in Table 2. The fermenters were maintained for 15 days with an average cell density of ~$2–3\times10^6$ cells/ml. As shown in Table 3, the addition of Pluronic F-68, copper, and trace metals significantly enhanced the specific productivity of BHK cells harboring the gene encoding factor VIII under protein-free conditions under continuous perfusion conditions. This production method can be easily adapted to larger fermenters (200 to 500 liter) equipped with cell retention devices such as settlers.

TABLE 3

Days	Specific Productivity (μU/cell/day) Basal Medium	Cu + metals
1	0.02	0.04
2	0.02	0.05
3	0.02	0.045
4	0.018	0.05
5	0.02	0.05
6	0.035	0.060
7	0.025	0.055
8	0.02	0.04
9	0.025	0.06
10	0.02	0.065
11	0.025	0.070
12	0.025	0.065
13	0.02	0.060
14	0.03	0.06
15	0.02	0.05

Additional Studies

The demonstrated that the expression of Factor VIII in recombinant BHK cells under protein-free conditions is significantly enhanced when cells were cultivated in the presence of polyol and copper sulfate. Factor VIII has been shown to contain a single copper atom, but the role of this metal in the structure and function of Factor VIII remains unclear. Recent reports show that Factor VIII subunits bind copper ions and suggest a functional role for copper in the assembly of the heavy and light chains of Factor VIII (Tagliavacca et al. 1, 1997; Sudhakar et al, 1998). We now further demonstrate that controlled amounts of copper ions alone (without polyols) are able to enhance the productivity of Factor VIII in recombinant BHK cells. Preferably, the copper is provided in the form of copper sulfate at a concentration ranging from about 50 to 800 nM in the culture medium and, very preferably includes the trace ions included in the above examples where polyols were used.

TABLE 4

Effect of Cu^{+2} on the production of FVIII in BHK cells under protein-free conditions.

Conditions	Titer (U/ml)	Specific Productivity (μU/cell/day)	% Increase over basal
Basal Medium	0.14	0.035	0.0000
Basal + Cu^{+2} (50 nM)	0.33	0.082	234
Basal + F-68	0.30	0.078	220
Basal + F-68 + Cu^{+2} (50 nM)	0.54	0.128	365

TABLE 5

Effect of various concentrations of Cu on the production of rFVIII in BHK cells under protein-free conditions.

Concentrations of Cu (nM)	Specific Productivity (μU/cell/day)
Basal Medium	0.040
20	0.062
50	0.078
100	0.080
200	0.082
400	0.080
800	0.081

The above examples are provided as a means of illustrating the present invention and are not to be construed as limiting the invention, which is solely defined by the claims.

References:

Bihoreau, N., et al., Eur. J. Biochem. 222: 41–48 (1994)
Chan, S. Y., U.S. Pat. No. 5,576,194 (1996)
Eis-Hubinger, A. M., et al., Thromb. Haemost. 76: 1120 (1996)
Mizrahi, A., J. Clin. Microbiol. 11–13 (1975)
Murhammer, D. W., et al. Biotechnol. Prog. 6: 142–148 (1990)
Papoutsakis, E. T., Trends in Biotechnology (Tibtech) 9: 316–324 (1991)
Qi, Y-M. et al., Cytotechnology 21: 95–109 (1996)
Schmolka, J. R., J. Am. Oil Chemists' Soc. 54: 110–116
Schneider, Y-J., J. Immunol. Meth. 116: 65–77 (1989)
Sudhakar, K., et al., Biochemistry 37: 6874–6882 (1998)
Tagliavacca, L., et al., J. Biol. Chem. 272: 27428–27434 (1997)
Wood, W., et al., Nature 312: 330–337 (1984)
Xu, D., et al., China J. Biotech. 11: 101–107 (1995)
Zhang, J., et al. Biotechnol. 33: 249–258 (1994)

We claim:

1. A method for production of recombinant Factor VIII from mammalian host cells carrying the cDNA coding for Factor VIII, comprising

(a) culturing said mammalian host cells in a medium free of plasma-derived protein and polyol supplements, said medium being supplemented with copper ions; and

(b) culturing for a time and under conditions effective to produce the factor VIII.

2. The method of claim 1 wherein the medium includes copper sulfate in an amount ranging from about 50 to about 800 nM.

3. The method of claim 2 wherein manganese ions are present in an amount ranging from about 1.5 to about 4.5 nM.

4. The method of claim 2 wherein ions containing molybdenum are present in an amount ranging from about 1.5 to about 4.5 nM.

5. The method of claim 2 wherein ions containing silicon are present in an amount ranging from 75 to about 300 nM.

6. The method of claim 2 wherein chromium ions are present in an amount ranging from about 1.0 to about 4.0 nM.

7. The method of claim 2 wherein lithium ions are present in an amount ranging from about 120 to about 480 nM.

8. The method of claim 1 wherein said mammalian host cell is selected from the group consisting of baby hamster kidney cells, human embryonic kidney cells, and Chinese hamster ovary cells.

9. A cell culture medium for the production of recombinant Factor VIII comprising a basal medium free of plasma-derived protein and including copper ions and insulin and which does not include polyols.

10. The medium of claim 9 wherein the copper ions are present in an amount ranging from about 50 to about 800 nM.

11. The medium of claim 10 including at least one trace metal selected from the group consisting of manganese, molybdenum, silicon, chromium and lithium.

* * * * *

EXHIBIT G

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(54) Title: COMPOSITION COMPRISING COAGULATION FACTOR VIII FORMULATION, PROCESS FOR ITS PREPARATION AND USE OF A SURFACTANT AS STABILIZER

(57) Abstract

The present invention relates to novel composition comprising coagulation factor VIII and a non-ionic surfactant such as block copolymers, e.g. polyoxamers or polyoxyethylene (20) sorbitan fatty acid esters e.g. polysorbate 20 or polysorbate 80 as stabilizer. The composition can also comprise sodium chloride, calcium chloride, L-histidine and/or sugars or sugar alcoh ls. The invention also relates to the use of a non-ionic surfactant as stabilizer for a composition comprising coagulation factor VIII.

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COMPOSITION COMPRISING COAGULATION FACTOR VIII FORMULATION, PROCESS FOR ITS PREPARATION AND USE OF A SURFACTANT AS STABILIZER.

The present invention relates to a novel formulation comprising coagulation factor VIII and a non-ionic surfactant such as block co-polymers, e.g. polyoxamers or polyoxyethylene (20) sorbitan fatty acid esters e.g. polysorbate 20 or polysorbate 80. The composition can also comprise sodium chloride, calcium chloride, L-histidine and/or sugars and/or sugar alcohols.

Haemophilia is an inherited disease which has been known for centuries but it is only within the last three decades that it has been possible to differentiate between the various forms; haemophilia A, haemophilia B and haemophilia C. Haemophilia A is the most frequent form. It affects only males with an incidence of one or two individuals per 10 000 live-born males. The disease is caused by strongly decreased level or absence of biologically active coagulation factor VIII (antihaemophilic factor) which is a protein normally present in plasma. The clinical manifestation of haemophilia A is a strong bleeding tendency and before treatment with factor VIII concentrates was introduced, the mean age of those patients was less than 20 years. Concentrates of factor VIII obtained from plasma have been available for about three decades. This has improved the situation for treatment of haemophilia patients considerably and given them possibility to live a normal life.

Therapeutic factor VIII concentrates have until now been prepared by fractionation of plasma. However, there are now methods available for production of factor VIII in cell culture using recombinant DNA techniques as reported in e.g. J Gitschier et al. Nature 312, 330-37 1984 and EP 160 457.

Factor VIII concentrates derived from human plasma contain several fragmented fully active factor VIII forms (Andersson et al, Proc. Natl. Acad. Sci. USA, Vol 83,2979-83, May 1986). The smallest active form has a

molecular mass of 170 kDa and consists of two chains of 90 kDa and 80 kDa held together by a metal ion bridge. Reference is here made to EP 197 901. Kabi Pharmacia has developed a recombinant factor VIII product which corresponds to the 170 kDa plasma factor VIII form in therapeutic factor VIII concentrates. The truncated recombinant factor VIII molecule is termed r-VIII SQ and is produced by Chinese Hamster Ovary (CHO) cells in a cell culture process in serum free medium at finite passage.

The specific activity of r-VIII SQ could be more than 12 000 IU/mg protein and preferably more than 14 000 IU/ mg. Activity of about 15 000 IU/mg has been measured. About 10 000 IU VIII:C per mg protein has earlier been known for our r-VIII SQ.

Recombinant factor VIII SQ is indicated for treatment of classical haemophilia. The dosage is similar to the dosage of the plasma factor VIII concentrates. Due to the high concentration now obtainable only small volumes are needed for injection.

The structure and biochemistry of recombinant factor VIII-products in general have been described by Kaufman Tibtech, Vol 9,1991 and Hematology, 63, 155-65, 1991. The structure and biochemistry of r-VIII SQ have been described in WO 91/09122.

The stability of proteins is generally a problem in pharmaceutical industry. It has often been solved by drying of the protein in different drying processes, such as freeze drying. The protein has thereafter been distributed and stored in dried form.
The solution before drying or freeze-drying, the dried material and the reconstituted product should all be stable, so that not too much activity is lost during the drying process, the storage or during handling.

Factor VIII which has been fractionated from plasma is normally sold as lyophilized powder which should be reconstituted with water.

A formulation with a low amount of protein will generally loose activity during purification, sterile manufacturing, in the package and during the administration. This problem is usually solved by the addition of human

albumin which reduces the activity loss of the active protein considerably. Human albumin functions as a general stabilizer during purification, sterile manufacturing and freeze-drying (see review by Wang et al., J. of Parenteral Sci. and Tech. Vol 42, Number 2S, supplement. 1988). Human albumin is also a good cake-former in a formulation for freeze-drying. The use of albumin for stabilization of factor VIII is known and is currently used in all highly purified factor VIII products on the market.
However, it is not desirable to add human albumin to a therapeutic protein manufactured by recombinant DNA technology. In addition, the use of human albumin as a formulation excipient often limits the use of many of the most powerful and sensitive analytical methods for protein characterization.

There is a need for albumin free formulations containing factor VIII and especially recombinant factor VIII which are stable during drying or freeze-drying, in solution and as a solution after reconstitution.

Several solutions have been proposed for the stabilization of different proteins:

EP 35 204 (Cutter) discloses a method for imparting thermal stability to a protein composition in the presence of a polyol.

EP 381 345 (Corint) discloses an aqeous liquid of a peptide, desmopressin, in the presence of carboxymethylcellulose.

In WO 89/09614 (Genentech), a stabilized formulation of human growth hormone comprising glycine, mannitol and a buffer is disclosed and in a preferred embodiment a non-ionic surfactant such as polysorbate 80 is added. The non-ionic surfactant is added for reduced aggregation and denaturation. The formulation has an increased stability in a lyophilized formulation and upon reconstitution.

EP 268 110 (Cetus) discloses a solution comprising a particular protein, interleukin-2, which is dissolved in an inert carrier medium comprising a non-ionic polymeric detergent as a solubilizer/stabilizer. The preferred

detergents are octylphenoxy polyethoxy ethanol compounds, poly thylene glycol monostearate compounds and polyethylene sorbitan fatty acid esters.

US 4 783 441 (Hoechst)discloses an aqueous solution comprising a protein, such as insulin and a surface active substance.

US 4 165 370 (Coval) discloses a gamma globulin solution and a process for the preparation thereof. The solutions contains polyethylene glycol (PEG). A non-ionic surfactant can be added to the solution.

In EP 77 870 (Green Cross) the addition of amino acids, monosaccarides, oligosaccarides or sugar alcohols or hydrocarbon carboxylic acid to improve stability of a solution containing factor VIII is disclosed and the addition of sugar alcohol or disaccarides to an aqueous solution of factor VIII for increasing stability during heat treatment has been disclosed in EP 117 064 (Green Cross).

WO 91/10439 (Octopharma) claims stable injectable solution of factor VIII or factor IX which comprises a disaccaride, preferably saccarose and one or more amino acids.

EP 315 968 and EP 314 095 (Rorer) claim stable formulations of factor VIII with different ionic strength.

Proteins are different with regard to physico-chemical properties. When preparing a pharmaceutical preparation which should be physico-chemical acceptable, and stable for a long time, consideration can not only be taken to the physiological properties of the protein but also other aspects must be considered such as the industrial manufacture, easy handling for the patient and safety for the patient. The results of these aspects are not predictable when testing different formulations and there often is a unique solution for each protein.

In plasma circulating factor VIII is stabilized by association with its carrier protein, the von Willebrand factor (vWF). In plasma and also in conventional intermediate purity factor VIII concentrates the ratio vWF to factor VIII is at least 50:1 on a weight basis. In very high purity factor VIII

concentrates, with a specific activity of more than 2 000 IU per mg protein, the ratio vWF to factor VIII is about 1:1 (w/w) and essentially all factor VIII is bound to vWF. Despite this stabilization further protection by the addition of albumin is required in order to achieve an acceptable stability during lyophilization and storage.

All super pure preparations on the market are stabilized with albumin (human serum albumin).

There is a now a demand for injectable factor VIII without albumin and containing a minimum of additives.

We have now developed a new formulation which solves the above mentioned problems for factor VIII.

To our great surprise we have found that factor VIII, which is a very sensitive protein, can be stabilized without albumin, when a non-ionic surfactant is added.

Thus the present invention relates to a composition comprising a coagulation factor VIII and a non-ionic surfactant as stabilizer. Our factor VIII is highly purified, i.e. has a specific activity of more than 5000 IU/mg protein, and the composition is stabilized without the addition of albumin.

When factor VIII is recombinant it can be either in its full-length form or as a deletion derivative such as SQ derivative.

The amount of factor VIII is from 10 to 100 000 IU/ml, preferably 50 to 10 000 IU/ml.

The non-ionic surfactant is preferably chosen from block co-polymers such as a poloxamer or polyoxyethylene (20) fatty acid ester, such as polysorbate 20 or polysorbate 80. Tween 80® has been used as polysorbate 80.

The non-ionic surfactant should be present in an amount above the critical micelle concentration (CMC). See Wan and Lee, Journal of Pharm Sci, 63, 136, 1974.

The polyoxyethylene (20) fatty acid ester is thus preferably in an amount of at least 0.01 mg/ml. The amount could e.g. be between 0.02 and 1 mg/ml.

The composition can also comprise sodium or potassium chloride, preferably in an amount of more than 0.1 M.

The composition comprises preferably a calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM and an amino acid such as L-histidine in an amount of more than 1 mM. The amount could e.g. be chosen between 0.05 and 500 mM. Mono-or disaccarides such as sucrose or sugar alcohols could be added e.g. in an amount of 1 to 300 mg/ml.

The composition comprises preferably L-histidine and sucrose. The ratio sodium chloride to L-histidine in the composition is preferably more than 1:1.

The composition could comprise
i) 10-100 000 IU/ml of recombinant factor VIII
ii) at least 0.01 mg/ml. of a polyoxyethylene (20) fatty acid ester
iii) sodium chloride, preferably in an amount of more than 0.1 M.
iv) calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM.
v) an amino acid such as L-histidine in an amount of more than 1 mM.

To this composition could mono-or disaccarides or sugar alcohols, preferably sucrose be added.
The composition could be in a dried form, preferably lyophilized or in aqeous solution before or after drying. The dried product is reconstituted with sterile water for injection or a buffer solution.

The claimed composition can also be a stable aqeous solution ready for use.

The invention also relates ot compositions in which the specific activity of r-VIII SQ is more than 12 000 IU / mg protein , preferably more than 14 000 IU / mg.

The claimed composition can be prepared by mixing factor VIII with a non-ionic surfactant in an aqeous solution, preferably together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt or by eluating factor VIII from the last purification step with a buffer containing a non-ionic surfactant in an aqeous solution, preferably together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt.

The invention also relates to the use of a non ionic surfactant preferably chosen from block co-polymers, preferably a poloxamer or polyoxyethylene (20) fatty acid ester, preferably polysorbate 20 or polysorbate 80, as stabilizer for a composition comprising coagulation factor VIII.

An amino acid is used to buffer the system and it protects also the protein in the amorphous phase. A suitable buffer could be L-histidine, lysine and/or arginine. L-Histidine has primarily been chosen because of the good buffer capacity of L-histidine around pH 7.

Sucrose or sugar alcohol can also be added for the protection of the protein.

Calcium (or divalent metal ions), here added as calcium chloride ($CaCl_2$) but other salts such as calcium gluconate, calcium glubionate or calcium gluceptate can also be used, is necessary for the maintenance of the association of factor VIII heavy and light chain.

The data presented in the examples indicate that r-VIII SQ is stable for at least 12 months when stored at 5±3°C.

The following examples illustrate the invention and show stability data for different formulations, all falling under the patent protection, a protection which is not limited to these examples.

The following figures are illustrating the invention:

Figure 1 HPLC gelfiltration, Example 10A, stored 5 months at 25°C.
Figure 2 HPLC gelfiltration, Example 10B, stored 5 months at 30°C.

EXPERIMENTAL

Material and methods

The production of recombinant factor VIII SQ (r-VIII SQ) was essentially performed as described in patent WO 91/09122, example 1-3. A DHFR deficient CHO celline (DG44N.Y.) was electroporated with an expression vector containing the r-VIII SQ gene and an expression vector containing the dihydrofolate-reductase gene. Following selection on selective media surviving colonies were amplified through growth in stepwise increasing amounts of methotrexate. Supernatant from the resulting colonies were individually screened for VIII:C activity. A production clone was chosen and this was subsequently adapted to serum free suspension growth in a defined medium and finally a large scale fermentation process was developed. Supernantant is collected after certain time periods and further purified as described below.

The clarified conditioned medium was pH adjusted and applied to a S-Sepharose FF column. After washing, factor VIII was eluated with a salt buffer containing 5 mM $CaCl_2$,.

Immunoadsorption was carried out on an immunoaffinity resin where the ligand was a monoclonal antibody (8A4) directed towards the heavy chain of Factor VIII. Before loading to the column the S-eluate was treated with 0,3 % TNBP and 1 % Octoxynol 9.
The column was equilibrated, washed and factor VIII was eluated with a buffer containing 0,05 M $CaCl_2$ and 50 % ethylene glycol.

The mAb-eluate was loaded on a Q-Sepharose FF column, equilibrated with the elution buffer in the immunoaffinity step. After washing, factor VIII was eluated with 0,05 M L-histidine, 4 mM $CaCl_2$, 0,6 M NaCl, pH 6,8.

The Q-eluate was applied to a gel filtration column (Superdex 200 p.g.). Equilibration and elution was carried out with a formulation containing sodium chloride, L-histidine, calcium chloride and polysorbate 80.

The protein peak was collected and the solution was formulated before freeze drying.

The VIII:C activity and the concentration of the inactive components were adjusted by diluting with an appropriate buffer. The solution was then sterile filtered (0,22 µm), dispensed and freeze-dried. Samples from each composition were frozen and stored at - 70 °C. These samples were thawed and used as references during the assay of VIII:C.

The coagulant activity VIII:C was assessed by a chromogenic substrate assay (Coatest Factor VIII, Chromogenix AB, Mölndal, Sweden). Activated factor X (Xa) is generated via the intrinsic pathway where factor VIII:C acts as cofactor. Factor Xa is then determined by the use of a synthetic chromogenic substrate, S-2222 in the presence of a thrombin inhibitor I-2581 to prevent hydrolysis of the substrate by thrombin. The reaction is stopped with acid, and the VIII:C, which is proportional to the release of pNA (para-nitroaniline), is determined photometrically at 450 nm against a reagent blank. The unit of factor VIII:C is expressed in international units (IU) as defined by the current International Concentrate Standard (IS) established by WHO.

The recovery of VIII:C is calculated as the percentage of VIII:C in the reconstituted solution divided by the VIII:C in the frozen and thawed solution for freeze-drying with appropriate adjustment for dilutions.

Soluble aggregates were determined by gel filtration. A prepacked Superdex 200 HR 10/30 column (Pharmacia) was used with a fluoroscence detector (exitation wavelength 280 nm), emission wavelength 340 nm). The reconstituted preparation were analysed. Evaluation of results from gelfiltration was done by visual examination of the chromatograms, or by integration of the peak areas if aggregates were found.

Recovery over freeze drying is expressed in % yield of frozen reference.

Example 1. Comparison between albumin and non-ionic surfactant.

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

The compositions were the following :

	1 A	1B	1 C	1D
L-Histidine, mM	50	50	50	50
Sodium chloride, M	0,6	0,6	0,6	0,6
Calcium chloride, mM	4	4	4	4
Polysorbate 80, %	-	-	0.02	-
PEG 4000, %	0.1	0.1	-	-
Albumin, %	-	1	-	1
VIII:C charged IU/ml	250	250	250	250
Recovery, IU/ml after reconstit.	83	197	232	222

This example shows that there was no difference in the recovery of factor VIII:C when the non ionic surfactant or albumin was used.

Example 2, Comparison between different strengths of non ionic surfactant

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 2 ml of sterile water for injections.

The compositions were the following :

	2 A	2B	2 C
L-Histidine/L-Glutamate equimolar amount, mg/ml	10	10	10
Sodium chloride, %	2	2	2
Calcium chloride, mg/ml	0.1	0.1	0.1
Polysorbate 80, %	-	0,001	0,01
VIII:C charged IU/ml	300	300	300
Recovery, IU/ml after reconstit.			
Initial	69	133	228
3.5 h*	43	140	222
7h*	49	133	204

* stored as reconstituted solution at ambient temperature

It is here clearly shown the surprisingly good stabilizing effect on factor VIII when a non ionic surfactant is used.

Example 3 , Variation of non-ionic surfactant concentration.

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	3 A	3B	3 C	3D	3E
L-Histidine, mM	50	50	50	50	50
Sodium chloride, M	0.34	0.34	0.34	0.34	0.34
Calcium chloride, mM	4	4	4	4	4
Polysorbate 80, %	0.01	0.02	0.03	0.04	0.05
Recovery, after reconstit., %	91	90	93	99	100

Results from this example indicate that the recovery of factor VIII (VIII:C) was very high after reconstitution and good for all concentrations of polysorbate 80 used.

Example 4. Variation of sodium chloride concentration

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized, stored at different temperatures for up to 6 months (mon) and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

		4 A	4B
L-Histidine.mM		50	50
Sodium chloride, M		0.3	0.6
Calcium chloride, mM		4	4
PEG-4000 % (Polyethylene glycol)		0.1	0.1
Polysorbate 80, %		0.025	0.025
Recovery , %, initial		85	86
stored at 8°C	3 mon	88	87
	4 mon	87	83
	6 mon	87	83
stored at 25°C,	1 mon	92	93
	3 mon	87	79
	4 mon	84	81
	6 mon	85	85
stored at 37°C	1 mon	88	90
	3 mon	80	80
	4 mon	80	77
	6 mon	81	80
stored at 50°C	1 mon	84	89
	3 mon	77	77
	4 mon	73	70

0,3 or 0,6 M sodium chloride showed very good stability. Both formulations were stable for 6 months at 37°C.

Example 5. Variation of L-Histidine concentration

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized, stored at different temperatures for up to 3 months (mon) and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

		5 A	5B
L-Histidine, mM		46	59
Sodium chloride, M		0.31	0.31
Calcium chloride, mM		3,7	3,7
PEG-4000 %		0.091	0.091
(Polyethylene glycol)			
Polysorbate 80, %		0.364	0.364
Recovery , %			
stored at 8°C,	Initial	78	84
	3 mon	70	76
stored at 25°C,	1 mon		
	3 mon	69	74
stored at. 37°C	1 mon	76	85
	3 mon	61	48
stored at 50°C	1 mon	60	73
	3 mon	44	48

This example shows that these different amounts of L-histidine does not effect the stability.

Example 6

Recombinant factor VIII was prepared according to the method described under Experimental.

	6A	6B
L-Histidine, mM	65	65
Sodium chloride. M	0.3	0.3
Calcium chloride, mM	4	4
PEG-4000 %	0	0.1
Tween 80, %	0.025	0.025

These solutions were freezed/thawed 1, 5 and 10 times and the recovery was the following:

	IU/ml	IU/ml
cold	298	291
1 freezing	293	293
5	295	287
10	290	288

These studies showed that VIII:C was stable after repeated freeze-thawing and that PEG-4000, which is thought to act as cryoprotectant, is not necessary in this formulation.

Example 7. Variation of pH

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	7 A	7B	7 C	7D
L-Histidine, mM	65	65	65	65
Sodium chloride, M	0,3	0,3	0,3	0,3
Calcium chloride, mM	4	4	4	4
Polysorbate 80, %	0.025	0.025	0.025	0.025
pH	6.0	6.5	7.0	7.5
Recovery, %, Initial	74	70	78	79
3 hours*	73	80	78	77

*stored as reconstituted solution at ambient temperature

This example shows that a pH is of no significant importance between 6.0 and 7.5 approx.

Example 8 Addition of sucrose

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	8A	8B
L-Histidine, mM	58	20.5
Sodium chloride. M	0.3	0.3
Calcium chloride, mM	3,7	3,7
Sucrose, mM	0	13.3
Polysorbate 80, %	0.025	0.025

Sucrose was added to the solution B after the final purification step before lyophilization.

The recovery after freeze-drying was 76 % for A and 87 % for B. The sam activity was found 4 hours after reconstitution stored at room temperature.

This study indicated that the addition of sucrose is favourable for the recovery of VIII:C over freeze-drying.

Example 9 . Variation of calcium salt

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	9 A	9B	9C	9D
L-Histidine, mM	23	23	23	23
Sodium chloride, M	0,34	0,34	0.34	0,34
Calcium chloride, mM	4	4	0.15	0.15
Polysorbate, %	0.025	0.025	0.025	0.025
Sucrose, mM	-	10	-	10
Calciumgluconate, mM	0	0	6	6
Recovery,%, Initial	63	74	74	78
4 hours*	60	73	73	77

*stored as reconstituted solution at ambient temperature

This example shows that $CaCl_2$ can be substituted by Calcium gluconate.

Example 10

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections. VIII:C per vial in reconstituted preparation was about 1000 IU.

	10A	10B
L-Histidine, mM	14.7	58
Sodium chloride. M	0.31	0.31
Calcium chloride, mM	3.7	3.7
Sucrose, mM	19.9	-
Polysorbate 80, %	0.025	0.025
Recovery, IU/ml after reconstitution		
Initial	213	198
4 h, 25 °C	213	198
24, 25 °C	201	182
Recovery , %		
Initial	92	91
5 months, 25°C	88	-
5 months, 30°C	76	85
12 months, 7°C	89	97

The recovery was good when part of the L-histidine was substituted by sucrose.

These formulations were studied by gelfiltration after 5 months' storage at 25°C and 30°C, respectively and the results are shown in figures 1 and 2. The only peaks to be seen is the peak at 42, indicating factor VIII:C and the peak at 70 which is histidine. Aggregates is to be found earlier than 40. From figure 1 it can be seen that no detectable amount of aggregates was found after 5 months at 25°C for 10A. Figure 2 shows a small amount of aggregates which is less than 2 % after 5 months at 30°C for 10B .

Example 11

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections. VIII:C per vial in reconstituted preparation was about 500 IU.

		11A	11B
L-Histidine, mM		14.7	58
Sodium chloride, M		0.31	0.31
Calcium chloride, mM		3.7	3.7
Sucrose, mM		19.9	-
Polysorbate 80, %		0.025	0.025
Recovery, IU/ml after reconstitution			
Initial		98	105
4 h, 25 °C		96	103
24, 25°C		93	101
Recovery , %			
Initial		91	93
stored at 25°C,	5 mon	89	87
stored at 30°C,	5 mon	76	79
stored at 7°C	12 mon	88	89

Both formulations showed good stability.

These formulations were studied by gelfiltration and the results were similar as shown in Figures 1 and 2.

No aggregation was formed when the formulations had been stored for 5 months at 25°C and 30°C, respectively.

Example 12

Recombinant factor VIII was prepared according to the method described under Experimental.

2 ml of the solution was lyophilized, stored at different temperatures for up to 3 months (mon) and thereafter reconstituted in an amount of 4 ml of sterile water for injections. VIII:C per vial in reconstituted preparation was about 500 IU.

	12A	12B
Mannitol, mg/ml	20	20
L-Histidine, mg/ml	2,67	2.67
Sodium chloride, mg/ml	18	18
Calcium chloride, mM	3,7	3,7
Polysorbate 80, mg/ml	0.23	0.23
Recovery , %		
initial	91	93
stored at. 7°C 5 mon	90	85

An acceptable stability was achieved after five months at 7°C.

CLAIMS

1. A composition comprising coagulation factor VIII and a non-ionic surfactant as stabilizer.

2. A composition according to claim 1 in which factor VIII is highly purified and stable without the addition of albumin.

3. A composition according to claim 1 or 2 in which factor VIII is full-length or a deletion derivative of recombinant factor VIII.

4. Composition according to any of claims 1-3 in which the amount of factor VIII is 10 to 100 000 IU/ml, preferably 50 to 10 000 IU/ml.

5. Composition according to any of claims 1-4 in which the non-ionic surfactant is present in an amount above the critical micelle concentration.

6. Composition according to any of claims 1-5 in which the non-ionic surfactant is chosen from block co-polymers, preferably a poloxamer or polyoxyethylene (20) fatty acid ester, preferably polysorbate 20 or polysorbate 80.

7. Composition according to claim 6 in which the polyoxyethylene (20) fatty acid ester is in an amount of at least 0.01 mg/ml.

8. Composition according to any of claims 1-7 which comprises sodium or potassium chloride, preferably in an amount of more than 0.1 M.

9. Composition according to any of claims 1-8 which comprises calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM.

10. Composition according to any of claims 1-9 which comprises an amino acid such as L-histidine in an amount of more than 1 mM.

11. Composition according to any of claims 1-10 which comprises mono-or disaccarides, prferably sucrose or sugar alcohols.

12. Composition according to any of claims 10-11 which comprises L-histidine and sucrose.

13. Composition according to claim 8 and 10 in which the ratio sodium chloride to L-histidine is more than 1:1.

14. Composition according to any of claims 1-13, comprising
i) 10-100 000 IU/ml of recombinant factor VIII
ii) at least 0.01 mg/ml. of a polyoxyethylene (20) fatty acid ester
iii) sodium chloride, preferably in an amount of more than 0.1 M.
iv) calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM.
v) an amino acid such as L-histidine in an amount of more than 1 mM.

15. Composition according to any of claims 1-14 which is dried.

16. Composition according to claim 15 which is lyophilized.

17. Composition according to any of claims 1-14 which is in a stable aqeous solution ready for use.

18. Composition according to any of claims 3-17 in which the specific activity of r-VIII SQ is more than 12 000 IU / mg protein , preferably more than 14 000 IU / mg.

19. Process for the preparation of the composition according to claim 1 characterized by mixing factor VIII with a non ionic surfactant in an aqueous solution, preferably together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt.

20. Process for the preparation of the composition according to claim 1 characterized by eluating factor VIII from the last purification step with a buffer containing a non-ionic surfactant in an aqeous solution, preferably

together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt.

21. Use of a non ionic surfactant preferably chosen from block co-polymers, preferably a poloxamer or polyoxyethylene (20) fatty acid ester, preferably polysorbate 20 or polysorbate 80, as stabilizer for a composition comprising coagulation factor VIII.

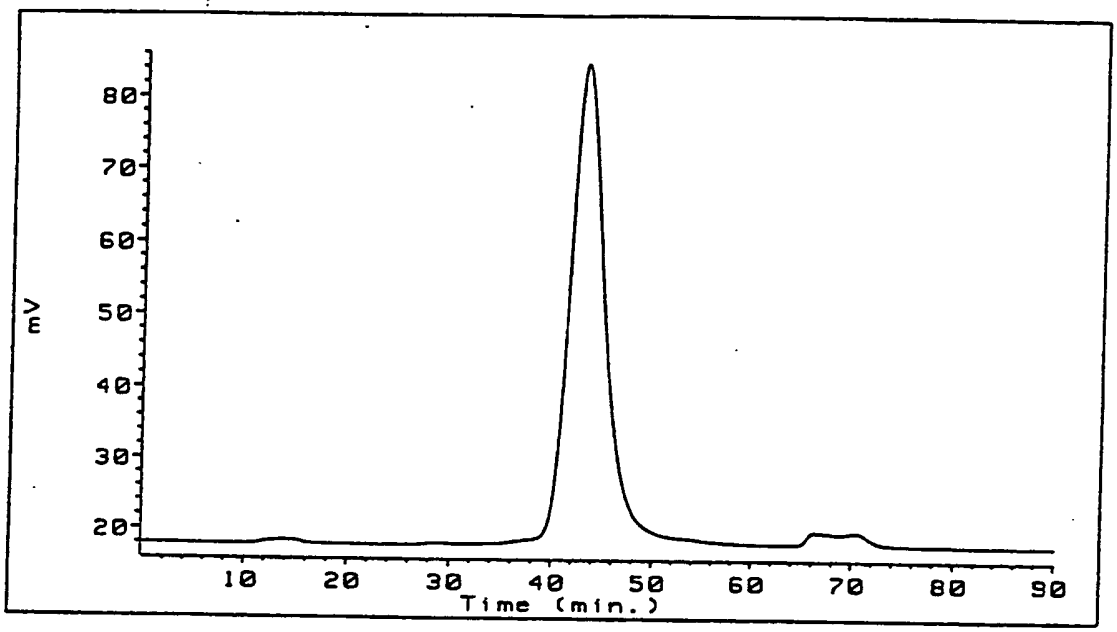

Figure 1

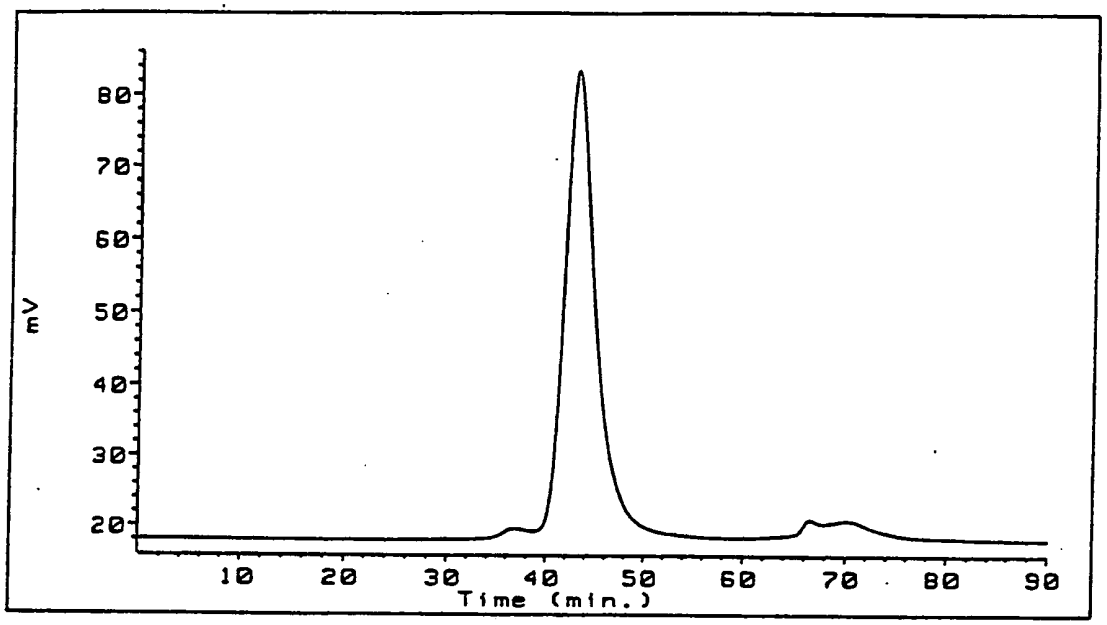

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 93/00793

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: A61K 35/16, A61K 37/02
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A61K, C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP, A1, 0508194 (BEHRINGWERKE AG), 14 October 1992 (14.10.92), see claim 6, examples 1-2	1-21
X	EP, A3, 0099445 (NEW YORK BLOOD CENTER, INC.), 1 February 1984 (01.02.84), see page 8, line 7 - line 14; page 19, line 24 - page 20, line 27	1-21
A	WO, A1, 9110439 (OCTA PHARMA AG), 25 July 1991 (25.07.91)	1-21

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 January 1994	12 -01- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Mikael G:son Bergstrand Telephone N . +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Informati n on patent family members

27/11/93

International applicati n No.
PCT/SE 93/00793

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0508194	14/10/92	AU-A- 1470292	15/10/92
		DE-A- 4111393	15/10/92
		JP-A- 5097702	20/04/93
EP-A3- 0099445	01/02/84	SE-T3- 0099445	
		AU-B- 561900	21/05/87
		AU-A- 1346283	20/10/83
		CA-A- 1207229	08/07/86
		JP-A- 58222023	23/12/83
		US-A- 4481189	06/11/84
		US-A- 4591505	27/05/86
WO-A1- 9110439	25/07/91	DE-A- 4001451	01/08/91
		EP-A- 0511234	04/11/92

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	F.C. Prats
Art Unit	:	1651
Applicant	:	Bruce Joseph Roser
Serial No.	:	08/875,796
Filed	:	October 30, 1998
For	:	Dried Blood Factor Compositions Comprising Trehalose

DECLARATION OF FRANCIS E. PRESTON UNDER 37 CFR 1.132

I, Francis E. Preston, hereby declare that:

My C.V. is attached.

I have read and understood the subject application, and the Office Action dated December 13, 2001. I have also reviewed the following references:

Curtis *et al.* (U.S. Patent No. 5,576,291, issued November 19, 1996)

Livesey *et al.* (U.S. Patent No. 5,364,756, issued November 15, 1994)

AND, being thus duly qualified, do further declare:

Factor VIII is a critical component in the human blood clotting process. Factor VIII deficiencies are responsible for haemophilia A which is a blood clotting disease afflicting a significant number of people. Through the administration of Factor VIII to those with haemophilia A, it is possible to minimize disability and to prolong life.

Unfortunately, obtaining sufficient quantities of Factor VIII to meet the demand for treating haemophilia patients has been very difficult. Factor VIII is present in low concentrations in blood plasma making it difficult to purify large quantities of Factor VIII. Also, plasma-derived blood factors carry a risk of transmitting viruses and other infectious agents.

Although the gene which encodes Factor VIII was identified in the mid-1980's, technical problems have hindered the ability to produce sufficient quantities of therapeutic preparations of

recombinant Factor VIII. One of the primary technical challenges is to stabilize the highly labile Factor VIII. Factor VIII is an extremely delicate protein, regardless of whether it is produced recombinantly or purified from plasma. Native Factor VIII contains multiple enzymatic cleavage sites making it highly susceptible to degradation. In the past, degradation of Factor VIII preparations has been avoided or minimized using albumin as a stabilizing agent.

Factor VIII purified from plasma necessarily contains albumin. Although the presence of albumin increases the chances for contamination with pathogens, albumin has been left in Factor VIII compositions purified from plasma because albumin was believed to be necessary to stabilize Factor VIII. Furthermore, until recently, a stabilizing amount of albumin was actually added to all therapeutic recombinant Factor VIII preparations. This practice has continued despite the potential health risks associated with albumin.

For years, it has been well known to those skilled in the art that Factor VIII, which is a large protein having over 2000 amino acids, is highly susceptible to degradation. In the human body, enzymes act on native Factor VIII during the clotting process. As the result of a complicated enzymatic conversion process, Factor VIII is transformed *in* vivo into a heterotrimer known as activated Factor VIII (Factor VIIIa). Specifically, proleolytic processing by thrombin results in the formation of Factor VIIIa which is, itself, a cofactor in the activation of Factor X by Factor IXa. Native Factor VIII is not a cofactor in the conversion of Factor X.

Thus, activated Factor VIII is a different chemical entity than Factor VIII. Activated Factor VIII has chemical, physical and physiological properties which all differ from Factor VIII. The scientific literature is replete with references to Factor VIII as well as to Factor VIIIa. The skilled artisan, in 1995, would be fully aware of the different nature of these compounds. The skilled artisan would also have been fully aware of the delicate nature of Factor VIII and the standard practice of stabilizing Factor VIII with albumin. At the time of the subject invention, those skilled in the art would recognize that "native Factor VIII" does not refer to activated Factor VIII.

Unlike Factor VIII, activated Factor VIII is not produced recombinantly. Rather, activated Factor VIII is produced by subjecting Factor VIII to proteolytic cleavage. This can be done, for example, by the process described by Curtis *et al.*

The Curtis *et al.* patent pertains to the administration of activated Factor VIII to treat a

particular complication of haemophilia. Curtis *et al.* intentionally proteolytically cleaves Factor VIII to obtain activated Factor VIII. This, of course, does not provide the skilled artisan with any information regarding the stability of Factor VIII, since the Factor VIII was purposefully degraded. I have not identified any disclosure in the Curtis *et al.* reference which would teach or suggest that native Factor VIII can be stabilized by trehalose in the absence of added albumin.

Finally, from my review of the Livesey *et al.* patent I again find no disclosure which would teach or suggest to a person skilled in this art that highly labile Factor VIII could be stabilized by trehalose in the absence of added albumin.

The undersigned declares further that all statements made herein of his own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements, and the like so made, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the Application or any Patent issuing thereon.

Further declarant sayeth naught

Signed: 

Date: 24.5.02

CURRICULUM VITAE

PROFESSOR FRANCIS ERIC PRESTON

Emeritus Professor of Haematology

MD, FRCP, FRC.Path

FEP/SLR
June 2002

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Professional Address:	Royal Hallamshire Hospital Department of Haematology Glossop Road, SHEFFIELD S10 2JF
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Specialty:	Haematology
Degrees:	MB ChB Liverpool 1963 MD (Liverpool) 1970 Jejunal Disaccharidases-Their Histopathological and Clinical Significance
Postgraduate Qualifications:	MRCPath Royal College of Pathologists 1970 FRCPath Royal College of Pathologists 1982 MRCP (By election, with citation) Royal College of Physicians, London 1987: FRCP Royal College of Physicians, London 1992

Current and Past Positions:

Professor of Haematology
University Department of Haematology
Royal Hallamshire Hospital
Glossop Road, SHEFFIELD
1986-

Director
Sheffield Comprehensive Care Haemophilia Centre
Royal Hallamshire Hospital
Glossop Road, SHEFFIELD
1973-2000

Consultant Haematologist
Royal Hallamshire Hospital
Glossop Road, SHEFFIELD
1973-

INTERNATIONAL APPOINTMENTS AND RESPONSIBILITIES

World Health Organisation

Director
WHO Collaborating Centre for the Diagnosis and Comprehensive Care of Patients with Bleeding and Clotting Disorders. This is only one of two such WHO-designated centres worldwide. 1994-2000

Director
WHO International External Quality Assessment Scheme in Blood Coagulation for Developing Countries (WHO IEQAS) 1994-current

Chairman
Co-ordinating Group of the WHO International External Quality Assessment Scheme (IEQAS) in Blood Coagulation 1997-current

International Society on Thrombosis and Haemostasis (ISTH)

Chairman
SSC Scientific Subcommittee on Control of Anticoagulation
1997-2000

Co-Chairman
SSC Scientific Subcommittee on Control of Anticoagulation
1996-current

Chairman
Working Party on Near Patient Testing and Self-Management of Oral Anticoagulant Control
1999-current

Member
Scientific and Standardization (SSC) Committee
1994-current

Member
SSC Working Group on Coagulation Standards
1995-current

Member
ISTH Scientific and Standardization Committee (SSC) Representative for WHO for the education and training in laboratory techniques for participants from developing countries

Member
International Advisory Committee for the XVIIth Congress of the International Society on Thrombosis and Haemostasis (ISTH), Washington, United States - 1999

Member
International Advisory Board for the XVIII Congress of the International Society on Thrombosis and Haemostasis (ISTH), Paris, July 6-12, 2001

World Federation of Haemophilia (WFH)

Director
WFH External Quality Assessment Scheme for Haemophilia Centres in Developing Countries 1993-current

Co-Chairman
WFH Laboratory Science Committee 1996-current

Director
WFH designated International Haemophilia Training Centre 1994-2000

Chairman
World Federation of Hemophilia Working Party on Chronic Liver Disease in Haemophilia
1990-1994

Member
Medical Advisory Panel 1988-1995

Member
WFH Visiting Faculty on Haemostasis and Thrombosis
to 1) Tianjin, China (1993) and 2) Jakarta, Indonesia (1994)

The British Council

Formal British Council link established between Sheffield and Recife, NE Brazil 1989-1994

Personally responsible for developing clinical and laboratory services in respect of Haemostasis and Thrombosis (including Haemophilia) 1989-1994

International Society of Haematology (ISH)

Member
Nomenclature Terminology, Quantity and Units Committee
1994-current

Member
Standing Committee on Education and Training
1996-current

European Prospective Cohort on Thrombosis (EPCOT)
This is an EC-funded European Collaborative Study in Familial Thrombophilia

Member
Steering Committee
1994-current

European Haematology Association

Member
1996-current

Stroke Prevention in Reversible Ischaemia Trial

Member
Advisory Committee of the European and Australian Stroke Prevention in Reversible Ischaemia Trial (ESPRIT)
1997-current

European Network on Oral Anticoagulant Treatment (ENAT)

Member
Board of ENAT
1997-current

External Examiner in Haematology for Master of Pathology Examination

University of Malaya, Kuala Lumpur
1988, 1992, 1995, 1999-2002

External Academic Appointments Assessor for Department of Pathology

University of Malaya, Kuala Lumpur
1988-1996
1999-2002

European Thrombosis Research Organisation (ETRO)

Member
Council of ETRO 1991-1994

Head
Elected ETRO Laboratory
1988-current

Elected UK Representative 1991-1994
Re-elected 1994-current

Member
Working Party on Familial Thrombotic Disorders

NATIONAL APPOINTMENTS AND RESPONSIBILITIES

Department of Health

Adviser to the Department of Health in respect of research requirements for individuals infected with chronic hepatitis C virus
1997

Department of Trade and Industry

Invited Member
Medical and Research Laboratories Interest Group (MERLIN)
1998

UK National External Quality Assessment Scheme (UK NEQAS)

Chairman
UK NEQAS for Blood Coagulation
1991-1992

Director
UK NEQAS Scheme for Blood Coagulation
1992-current

British Society for Haematology (BSH)

President
1995/96

Elected Committee Member
1984-1987

Committee Member (As representative of the British Society for Haematology (BSHT)
1989-1992

Member
Haemostasis and Thrombosis Task Force
1985-current

Member
British Committee for Standardisation in Haematology
1985-1988

Member
Subcommittee on European Matters
1996-current

British Society for Haemostasis and Thrombosis

President
1988-1989

Honorary Secretary
1983-1988

Committee Member
1982-1990

British Atherosclerosis Society
(Formerly MRC Atherosclerosis Discussion Group)

Member
1997-current

Royal College of Pathologists

Elected Fellow
1982

Member
Haemostasis Subcommittee of the Standing Advisory Committee on Haematology
1987-1991

Member
Standing Advisory Committee on Haematology
1991-1994

Member
Panel of Examiners for Haematology
1987-current

College Assessor in Coagulation and Haemophilia

Member
Visiting Teaching Group to Jeddah, Saudi Arabia 1996

Association of Clinical Pathologists

Member
1972-

British Heart Foundation

Member
Research Funds Committee 1988-1991

UK Haemophilia Society

Member
Medical Advisory Board
1989-current

UK Haemophilia Organisation

Member
UK Haemophilia Reference Centre Directors Organisation

Chairman
Committee of the Working Party on Congenital Platelet Disorders

Chairman
Chronic Liver Disease in Haemophilia Working Party
1990-1999

Member
Haemophilia Reference Centre Directors AIDS Group

Member
Factor VIII Inhibitor Working Party
1995-current

Principal Editor

Platelets Journal -(1989)

Editorial Boards

Member
Thrombosis and Haemostasis
1998-2004

Member
Blood Coagulation and Fibrinolysis Journal
1989-current

Member
Haemophilia Journal
1994-current

Member
Hemophilia Forum
1997-current

Member
The Hematology Journal
1999-

Review Editor

International Monitor of Hemophilia
1993-current

University of Sheffield

Member
Faculty Board

Member
Fourth Year Teaching Committee

European Community (EC) Representative for the Department of Haematology

Head of Section of Haematology
1996-2000

Member
Division of Molecular and Genetic Medicine Strategy & Planning Task Group

Trent Regional Health Authority

Member
Ad hoc Committee on AIDS

Member
Blood Borne Viruses Group

Member
Genetics Review Group

Sheffield Health Authority

Member
Advisory and Monitoring Group (AIDS and HIV)

Sheffield Teaching Hospitals
Royal Hallamshire Hospital, Sheffield

Member
AIDS Working Party

External Examiner for PhD/MD Theses

Universities of Nottingham, Southampton, London
Bath, Leeds, Aberdeen, Glasgow and Manchester

External Examiner for MB.BS

University of London Collegiate Committee of Examiners for examinations in clinical studies and related sciences for the M.B., B.S. Degrees

United Medical & Dental Schools of Guy's and and St Thomas's Hospitals, London

MAIN PUBLICATIONS

1) F E Preston, R Christenhusz
Microangiopathic haemolytic anaemia: The phenomenon of red cell adherence.
Journal of Clinical Pathology 1970; 23; 711-714

2) H Themann, F E Preston, D M Roberts
Electron microscope findings in the jejunal mucosa of patients with psoriasis
Arch Klin Dermatology 1970; 238; 323

3) D M Roberts, H Themann, F-J Knust, F E Preston, J R Donaldson
An electron-microscopic study of bacteria in two cases of Whipple's disease
Journal of Pathology 1970; 100; 249-255

4) F E Preston, R T Shahani
Surface ultramicroscopy of neonatal erythrocytes
The Lancet 1970; 1; 1177-1178

5) F E Preston, D Lee
The effects of phenothiazines on human erythrocytes: An ultrastructural study.
British Journal of Haematology 1971; 20; 563-566

6) F E Preston, R G Malia, E K Blackburn, M Platts
Fibrin degradation products in glomerulonephritis
British Medical Journal 1971; iv; 171-172

7) D M Roberts, F E Preston
Intestinal disaccharides activity in psoriatic enteropathy
Scandinavian Journal of Gastroenterology 1971; 6; 93-96

8) F E Preston, R G Malia, R H Tipton, A J Smith
Intravascular coagulation and pre-eclamptic toxaemia
The Lancet 1972; i; 34-35

9) F E Preston, D Lee
Fibrinolysis in myelomatosis
Acta Haematologica 1972; 47; 65-70

10) F E Preston, A Milford-Ward
Acute renal failure in myelomatosis from intravascular coagulation
British Medical Journal 1972; i; 604-606

11) F E Preston, I R Edwards
Post-partum purpura fulminant: Successful management with Streptokinase
British Medical Journal 1973; iii; 329-330

12) M J Sworn, F E Preston, F McGinty
Disseminated intravascular coagulation with thymoma-associated Cushing's syndrome
British Medical Journal 1973; ii; 343-345

13) F E Preston, R G Malia, M J Sworn, E K Blackburn
Intravascular coagulation and E Coli Septicaemia
Journal of Clinical Pathology 1973; 26; 120-125

14) F E Preston, W R Timperley, J D Ward
Diabetic ketoacidosis presenting as neurosurgical emergencies
British Medical Journal 1974; ii; 341-342

15) W R Timperley, F E Preston, J D Ward
Cerebral intravascular coagulation in diabetic ketoacidosis
The Lancet 1974; i; 952

16) F P Ryan, R C Smart, F E Preston, C D Holdsworth
Hyposplenism in ulcerative colitis
The Lancet 1974; ii; 318-320

17) F E Preston, W R Timperley, R G Malia, E K Blackburn
Disseminated intravascular coagulation as a consequence of cerebral damage
Journal of Neurological Psychiatry 1974; 37; 241-248

18) F E Preston, I G Emmanuel, D A Winfield, R G Malia
Essential thrombocythaemia and peripheral gangrene
British Journal of Medicine 1974; iii; 548-552

19) A Milford-Ward, F E Preston
The kidney and intravascular coagulation in myelomatosis
British Medical Journal 1974; ii; 529-531

20) F E Preston, D A Winfield, R G Malia, E K Blackburn
Serial changes in the coagulation system following clotting factor concentrate infusion
Thrombosis Diathesis Haemorrhag 1975; 34; 475-482

21) B C O'Malley, W R Timperley, J D Ward, N R Porter, F E Preston
Platelet abnormalities in diabetic peripheral neuropathy
The Lancet 1975; ii; 1274-1276

22) I Cook, M Foley, E Lewton, F E Preston
The treatment of puerperal lactation with Bromocriptine
Postgraduate Medical Journal 1976; 52; 75-80

23) W R Timperley, J D Ward, F E Preston, T Duckworth, B C O'Malley
Clinical and histological studies in diabetic neuropathy
Diabetologia 1976; 12; 237-243

24) F E Preston, R C W Dinsdale, D J Sutcliffe, G Bardhan, P J Wyld, J F Hamlyn
Factor VIII inhibitor bypassing activity (FEIBA) in the management of patients with factor VIII inhibitors
Thrombosis Research 1977; 11; 643-651

25) F E Preston, R G Malia, J S Lilleyman, E K Blackburn
Heparinised clotting factor concentrates in patients with Christmas disease and liver disease
Thrombosis Haemostasis (Stuttgart) 1977; 38; 504-509

26) L C Best, T J Martin, R G G Russell, F E Preston
Prostacyclin increases cyclic AMP levels and adenylate cyclase activity in platelets
Nature 1977; 267; 850-851

27) S Pritchard, J Watkins, A Murphy, P J Wyld, F E Preston, G Hudson
A simple technique for studying immunoglobulin synthesis by normal and malignant plasma cells in vitro
Journal of Immunological Methods 1977; 17; 211-216

28) F P Ryan, W R Timperley, F E Preston, C D Holdsworth
Cerebral involvement with disseminated intravascular coagulation in intestinal disease
Journal of Clinical Pathology, 1977; 30; 551-555

29) F E Preston, R J Sokol, J S Lilleyman, D A Winfield
Cellular hyperviscosity as a cause of neurological symptoms in leukaemia
British Medical Journal 1978; i; 476-478

30) F E Preston, J D Ward, H B Marcola, N R Porter, W R Timperley, B C O'Malley
Elevated beta-thromboglobulin levels and circulating platelet aggregates in diabetic microangiopathy
The Lancet 1978; i; 238-240

31) F P Ryan, R C Smart, C D Holdsworth, F E Preston
Hyposplenism in inflammatory bowel disease
Gut 1978; 19; 50-55

32) L C Best, T J Martin, M B McGuire, F E Preston, R G G Russell, D S Segal
Dipyridamole and platelet function
The Lancet 1978; ii; 846

33) D A Lane, F E Preston, M E Van Ross, V V Kakkar
Characterisation of serum fibrinogen and fibrin fragments produced during disseminated intravascular coagulation
British Journal of Haematology 1978; 40; 609-615

34) A S Ward, N Porter, F E Preston, W Morris-Jones
Platelet aggregation in patients with peripheral vascular disease
Atherosclerosis 1978; 29; 63-68

35) H Connor, D J Newton, F E Preston, H F Woods
Oral methionine loading as a cause of acute serum folate deficiency: Its relevance to parenteral nutrition
Postgraduate Medical Journal 1978; 54; 318-320

36) F E Preston, K B Cooke, E M Foster, D A Winfield, D Lee
Myelomatosis and the hyperviscosity syndrome
British Journal of Haematology 1978; 38; 517-530

37) D S Pepper, F E Preston, R S Mibashan
Newer techniques in the study of haemostatic disorders.
Seminars in Haematology 1978; 15; 73-92

38) R C Smart, F P Ryan, C D Holdsworth, F E Preston
The relationship between splenic size and splenic function
Gut 1978; 19; 56-59

39) F E Preston et al
Percutaneous liver biopsy and chronic liver disease in haemophiliacs
The Lancet 1978; ii; 592-594

40) F E Preston
Haematological problems associated with shock
British Journal of Hospital Medicine 1979; 21; 232-245

41) F E Preston, J F Martin, R M Stewart, G A B Davies-Jones
Thrombocytosis circulating platelet aggregates and neurological dysfunction
British Medical Journal 1979; 2; 1561-1563

42) F E Preston
Hyperviscosity and other complications of para-proteinaemia
Journal of Clinical Pathology 1979; 32 Suppl.13; 85-89

43) L C Best, M B McGuire, P B B Jones, T K Holland, T J Martin,
F E Preston, D S Segal, R G G Russell
Mode of action of Dipyridamole on human platelets
Thrombosis Research 1979; 16; 367-479

44) L C Best, P B B Jones, F E Preston
Effect of glucose on platelet thromboxane biosynthesis
The Lancet 1979; ii; 790

45) L C Best, M B McGuire, T J Martin, F E Preston, R G G Russell
Effects of epoxymethane analogues of prostaglandin endoperoxides on aggregation and release of 5-HT and on the metabolism of 3'5'-cyclic AMP and cyclic GMP in human platelets
Biochimica Biophys.Acta 1979; 583; 344-351

46) R G Malia, B M Sampson, S Tomlinson, F E Preston
An immunoradiometric assay for porcine factor VIII-related antigen. Thrombosis Research 1980; 19; 561-570

47) D L Douglas, T Duckworth, R G G Russell, J A Kanis, C J Preston, F E Preston, M A Prenton, J S Woodhead
The effect of dichloronethylene diphosphonate in Paget's disease of bone and in hypercalcaemia due to primary hyperparathyroidism or malignant disease
The Lancet 1980; i; 1043-1048

48) R G Malia, F E Preston, V E Mitchell
Evidence against vitamin K deficiency in normal neonates
Thrombosis and Haemostasis 1980; 44; 159-160

49) R J Davies, D A Lane, I R McGregor, F E Preston
The neutralisation of heparin by Trasylol
Thrombosis Research 1980; 17; 533-537

50) F E Preston
The effect of stanozolol on the fibrinolytic enzyme system in normal subjects
Scottish Medical Journal 1981; Suppl.; S59-S63

51) F E Preston
Haematological consequences of shock
Clinical Research Reviews 1981; 1; 51-54

52) W Morris-Jones, F E Preston, M Greaney, D K Chatterjee
Gangrene of the toes with palpable peripheral pulses: response to platelet suppressive therapy
Annals of Surgery 1981; 193; 462-464

53) R G F Gray, A D Patrick, F E Preston, M F Whitfield
Acute hereditary tryosinaemia type I clinical, biochemical and haematological studies in twins
Journal of Inherited Metabolic Disorders 1981; 4; 37-40

54) F E Preston, B K Burakowski, N R Porter, R G Malia
The fibrinolytic response to stanozolol in normal subjects
Thrombosis Research 1981; 22; 543-551

55) F E Preston, M Greaves, C A Jackson
Cumulative inhibitory effect of daily 40mg aspirin on prostacyclin synthesis
The Lancet 1981; i; 1211-1212

56) F E Preston, S Whipps, C A Jackson, A J French, P J Wyld,
C J Stoddard
Inhibition of prostacyclin and platelet thromboxane A2 after low-dose aspirin
New England Journal of Medicine 1981; 304; 76-79

57) K Shinton, A L Bloom, B Colvin, P T Flute, F E Preston, D A Kennedy
Tentative protocol for the evaluation of coagulometers based on one-stage prothrombin time
Clinical Laboratory Haematology 1981; 3; 71-76

58) M Greaves, F E Preston
Plasma 6-keto-prostaglandin F1-alpha: fact or fiction?
Thrombosis Research 1982; 26; 145-157

59) M Greaves, F E Preston
Prostacyclin and thromboxane A2.
British Medical Journal 1982; 284; 419

60) C A Jackson, M Greaves, A D Patterson, C B Brown, F E Preston
Relationship between platelet aggregation, thromboxane synthesis and albumin concentration in nephrotic syndrome
British Journal of Haematology 1982; 52; 69-77

61) F E Preston, M Greaves, C A Jackson, C J Stoddard
Low-dose aspirin inhibits platelet and vessel wall cyclo-oxygenase in man
Thrombosis Research 1982; 27; 477-484

62) F E Preston
Disseminated intravascular coagulation
British Journal of Hospital Medicine 1982; 28; 129-137

63) C A Jackson, M Greaves, F E Preston
A study of the stimulation of human venous prostacyclin synthesis by Dipyridamole
Thrombosis Research 1982; 27; 563-573

64) F E Preston
Disorders of haemostasis in diabetes mellitus
La Ricerca 1982; XII; 425-438

65) F E Preston, R G Malia, M Greaves, G Wijngaards, C Kluft
The effect of stanozolol on thrombotic risk factors in healthy individuals
Progress in Fibrinolysis 1983; 6; 519-523

66) F E Preston
Aspirin, prostaglandins and peripheral gangrene
American Journal of Medicine 1983; 74; 55-60

67) A D Paterson, A Kanis, E C Cameron, D L Douglas, D J Beard,
F E Preston, R G G Russell
The use of dichloromethylene diphosphonate for the management of hypercalcaemia in multiple myeloma
British Journal of Haematology 1983; 54; 121-132

68) R J Johnson, X P Zhu, I Isherwood, A I Morris, B A McVerry,
D R Triger, F E Preston, S B Lucas
Computer tomography: qualitative and quantitative recognition of liver disease in haemophilia
Journal of Computer Assisted Tomography 1983; 7; 1000-1006

69) B E Woodcock, F E Preston
Haemorrhage and factor IX deficiency in pituitary insufficiency
Acta Haematologica 1983; 70; 205-206

70) C Kluft, F E Preston, R G Malia, G Wijngaards
Characterisation of the effect of stanozolol on fibrinolytic parameters in healthy volunteers
Progress in Fibrinolysis 1983; 6; 513-518

71) B M Sampson, M Greaves, R G Malia, F E Preston
Acquired von Willebrand's disease: Demonstration of a circulating inhibitor of the factor VIII complex in four cases
British Journal of Haematology 1983; 54; 233-244

72) F E Preston, R G Malia, M Greaves, C Kluft, R M Bertina, D S Segal
Effect of stanozolol on antithrombin III and Protein C
The Lancet 1983; ii; 517-518

73) C Kluft, R M Bertina, F E Preston, R G Malia, S L Blamey,
G D O Lowe, C D Forbes
Protein C, an anticoagulant protein is increased in healthy volunteers and surgical patients after treatment with stanozolol
Thrombosis Research 1984; 33; 297-304

74) B E Woodcock, E Smith, W H Lambert, W Morris-Jones,
J H Galloway, M Greaves, F E Preston
Beneficial effect of fish oil on blood viscosity in peripheral vascular disease.
British Medical Journal 1984; 288; 592-594

75) M Greaves, B E Woodcock, J H Galloway, F E Preston
Studies on the incorporation of eicosapentaenoic acid (EPA) into platelet membrane phospholipids and the effects of EPA supplementation on platelet function, skin bleeding time and blood viscosity in man.
British Journal of Clinical Practice 1984; 38; 45-48

76) N D S Bax, M S Lennard, G T Tucker, H F Woods, N R Porter,
R G Malia, F E Preston
The effect of beta-adrenoceptor antagonists on the pharmacokinetics and pharmacodynamics of Warfarin after a single dose
British Journal of Clinical Pharmacology 1984; 17; 553-557

77) C Kluft, F E Preston, R G Malia, R M Bertina, G Wijngaards,
M Greaves, J H Verheijen, G Dooijewaard
Stanozolol-induced changes in fibrinolysis and coagulation in healthy adults.
Thrombosis and Haemostasis 1984; 51; 157-164

78) C Patrono, F E Preston, J Vermylen
Platelet and vascular arachidonic acid metabolites; can they help detect a tendency towards thrombosis?
British Journal of Haematology 1984; 57; 209-212

79) C A Jackson, M Greaves, A J M Boulton, J D Ward, F E Preston
Near-normal glycaemic control does not correct abnormal platelet reactivity in diabetes mellitus.
Clinical Science 1984; 67; 551-555

80) J H Verheijen, D C Rijken, G T C Chang, F E Preston, C Kluft
Modulation by rapid plasminogen activator inhibitor in plasma by stanozolol
Thrombosis and Haemostasis 1984; 51; 396-397

81) B E Woodcock, P C Cooper, P R Brown, C Pickering, D A Winfield,
F E Preston
The platelet defect in acute myeloid leukaemia
Journal of Clinical Pathology 1984; 3; 1339-1342

82) F E Preston, M Greaves
Thrombotic vascular disease
British Journal Hospital Medicine 1985; 3; 46-50

83) A Bloom, P A Hannaford, M Greaves, F E Preston, C B Brown
Hemolytic-uremic syndrome demonstration of abnormalities of platelet reactivity and insensitivity to prostaglandin L2
Clinical Nephrology 1985; 2; 85-88

84) J H Galloway, I J Cartwright, B E Woodcock, M Greaves,
R G G Russell, F E Preston
Effects of dietary fish oil supplementation on the fatty acid composition of the human platelet membrane; demonstration of selectivity in the incorporation of eicosapentaenoic acid into membrane phospholipid pools
Clinical Science 1985; 6; 449-454

85) I Greer, M Greaves, R Hadkok, K McLoughlin, N R Porter,
G D O Lowe, F E Preston, C D Forbes
Effect of stanozolol on factors VIII and IX on serum aminotransferases in haemophilia
Thrombosis and Haemostasis 1985; 53; 386-389

86) I J Cartwright, A G Pockley, J H Galloway, M Greaves, F E Preston
The effects of dietary w-3 polyunsaturated fatty acids on erythrocyte membrane phospholipids, erythrocyte deformability and blood viscosity in healthy volunteers.
Atherosclerosis 1985; 55; 267-281

87) C R M Hay, F E Preston, D R Triger, J C E Underwood
Progressive liver disease in haemophilia: an understated problem?
The Lancet 1985; i; 1495-1498

88) F E Preston, M Greaves
Platelet suppressive therapy in clinical medicine
British Journal of Haematology 1985; 60; 589-597

89) D M Reardon, D Hutchinson, F E Preston, E A Trowbridge
The routine measurement of platelet volume; a comparison of aperture-impedance and flow cytrometric systems
Clinical and Laboratory Haematology 1985; 7; 251-257

90) C A Carne, A Smith, S G Elkington, F E Preston, R S Tedder,
S Sutherland, H M Daly, J Craske
Acute encephalopathy coincident with sero-conversion for anti-HTVLIII
The Lancet 1985; i; 1206-1208

91) C Kluft, J H Verheijen, A F H Jie, D C Rijken, F E Preston,
H M Sue-Ling, J Jespersen, A O Aasen
The post-operative fibrinolytic shutdown: a rapidly reverting acute phase pattern for the fast-acting inhibitor of tissue-type plasminogen activator after trauma
Scandinavian Journal of Clinical Laboratory Investigations 1985; 45; 605-610

92) M Greaves, F E Preston, C Kluft, R M Bertina, R G Malia, M Simms
A comparative study of the influence of intramuscular stanozolol and subcutaneous heparin on coagulation and fibrinolysis following abdominal surgery
Progress in Fibrinolysis 1985; 7; 105-107

93) P J Lawson-Matthew, J C Clayton, D F Guilland-Cumming,
A J F Yates, R G G Russell, F E Preston, J A Kanis
Defective production of 1,25 (OH) 2D3 in Myeloma, vitamin D
Chemical Biochemical and Clinical Update 1985; 891-892

94) J H Verheijen, D C Rijken, G T G Chang, F E Preston, C Kluft
Decreased level of rapid tissue-type plasminogen activator inhibition by oral administration of stanozolol
Progress in Fibrinolysis 1985; 7; 102-104

95) M Greaves, F E Preston
The laboratory investigation of acquired and congenital platelet disorders
Blood Coagulation and Haemorheology 1985; 6; 373-379

96) C R M Hay, F E Preston, D R Triger, J C E Underwood, J S Lilleyman,
Chronic liver disease in haemophilia: the emerging natural history
British Journal of Haematology 1985; 61(3); 568-569

97) B K Park, I A Choonara, B Haynes, A Breckenridge, R G Malia,
F E Preston
Abnormal vitamin K metabolism in the presence of normal clotting factor activity in factory workers exposed to 4-Hydroxycoumarin
British Journal of Clinical Pharmacology 1986; 21; 289-294

98) C Carter, M Greaves, F E Preston
Orbital plasmacytoma in established myeloma - a report of two cases
Clinical and Laboratory Haematology 1986; 155-159

99) A G Pockley, P Davenport, F Paul, M Greaves, F E Preston
The use of high gradient magnetic separation for preparation of leucocyte and platelet free red cell suspensions
Clinical Haemorheology 1986; 6; 373-379

100) Aids Group of the UK Haemophilia Centre Directors
Prevalence of antibody to HTLV III in haemophiliacs in the United Kingdom
British Medical Journal 1986; 293; 175-176

101) M Couch, F E Preston, R G Malia, R Graham, G Russell
Changes in plasma osteocalcin concentration following treatment with stanozolol
Clinica Chimica Acta 1986; 158; 43-47

102) M Greaves, C Pickering, J Martin, I J Cartwright, F E Preston
A new familial giant platelet syndrome with structural metabolic and functional abnormalities of platelets due to a primary megakaryocyte defect
British Journal of Haematology 1986; 63; 199-200

103) J Ball, R G Malia, M Greaves, F E Preston
Demonstration of abnormal factor VIII multimers in acquired von Willebrand's disease associated with a circulating inhibitor
British Journal of Haematology 1987; 65; 95-100

104) C R M Hay, P C Waller, C Carter, H A Cameron, L Parnell,
L E Ramsay, F E Preston, M Greaves
Lack of effect of a 24-hour infusion of Iloprost in intermittent claudication
Thrombosis Research 1987; 46; 317-324

105) R C Collin, M Greaves, F E Preston
Potential value of vinchristine-adriamycin-dexamethasone combination chemotherapy (VAD) in refractory and rapidly progressive myeloma
Cur. Journal of Haematology 1987; 39; 203-208

106) M Greaves, C Pickering, G Knight, A J M Boulton, J Ball, J D Ward, F E Preston
Changes in the factor VIII complex in diabetic ketoacidosis; evidence of endothelial cell damage
Diabetologia 1987; 30; 160-165

107) R G Dalton, M S Dewar, G F Savidge, P B A Kernoff, K B Matthews, M Greaves, F E Preston
Hypothyroidism as a cause of acquired von Willebrand's disease
The Lancet 1987; 1007-1009

108) C R M Hay, F E Preston, D R Triger, M Greaves, J C E Underwood, L Westlake
Predictive markers of chronic liver disease in haemophilia
Blood 1987; 69; 1595-1599

109) G H Urwin, A J P Yates, R E S Gray, N A T Hamdy, E V McCloskey, F E Preston, M Greaves, F E Neil, J A Kanis
Treatment of the hypercalcaemia of malignancy with intravenous clodronate
Bone 1987; 8; S43-S51

110) L Williamson, J Johnstone, F E Preston
Microvascular thrombosis of the bowel in paroxysmal nocturnal haemoglobulinurea
Journal of Clinical Pathology 1987, 40; 930-931

111) P B A Kernoff, E J Miller, G F Savidge, S J Machin, M S Dewar,
F E Preston
Reduced risk of non-A, non-B hepatitis after a first exposure to 'wet-heated' factor VIII concentrate
British Journal of Haematology 1987; 67(2); 207

112) I J Cartwright, M Greaves, F E Preston
Abnormal platelet reactivity in diabetic microvascular disease: is thromboxane synthesis related to platelet arachidonic acid content?
Agent Action 1987; 22; 364

113) J T Wilde, K M Roberts, M Greaves, F E Preston
Association between necropsy evidence of disseminated intravascular coagulation and coagulation variables before death in patients in intensive care units
Journal of Clinical Pathology 1988; 41; 138-142

114) I A Choonara, R G Malia, B P Haynes, C R M Hay, S Chloerton,
A M Breckenridge, F E Preston, B K Park
The relationship between inhibition of vitamin K1 2,3-epoxide reductase and reduction of clotting factor activity with Warfarin
British Journal of Clinical Pharmacology 1988; 25; 1-7

115) S Kitchen, R G Malia, M Greaves, F E Preston
A method for the determination of activated factor VII using bovine and rabbit brain thromboplastins: demonstration of increased levels in disseminated intravascular coagulation
Thrombosis Research 1988; 50; 191

116) J Dawes, D A Pratt, M S Dewar, F E Preston
Do extra platelet sources contribute to the plasma level of thrombospondin?
Thrombosis and Haemostasis 1988; 59(2); 273-276

117) J T Wilde, W E G Thomas, D A Lane, M Greaves, F E Preston
Acquired dysfibrinogenaemia masquerading as disseminated intravascular coagulation in acute pancreatitis
Journal of Clinical Pathology 1988; 41; 615-618

118) J G Wright, P Cooper, B Astedt, I Lecander, J T Wilde, F E Preston, M Greaves
Fibrinolysis during normal human pregnancy; complex inter-relationships between plasma levels of tissue plasminogen activator and inhibitors on the euglobulin clot lysis time
British Journal of Haematology 1988; 69; 253-258

119) J T Wilde, H Ireland, D A Lane, M Greaves, F E Preston
The relationship between plasma D-dimer and serum fragment E levels in hypercoaguable states
Fibrinolysis 1988; 2 Suppl.1; 36-40

120) G Dolan, M Greaves, F E Preston
Familial plasminogen deficiency in thrombovascular disease
Fibrinolysis 1988; 2 Suppl.1; 97-99

121) F E Preston, W Morris-Jones, C Brookfield, P Cooper
Reduced fibrinolytic activity in lipodermatosclerosis
Fibrinolysis 1988; 2 Suppl.1; 172-176

122) G Dolan, F E Preston
Familial plasminogen deficiency in thromboembolism
Fibrinolysis 1988; 2 Suppl.2; 26-34

123) G Dolan, M Greaves, F E Preston
Type 1 plasminogen deficiency in thrombovascular disease in three kindreds
Fibrinolysis 1988; 2 Suppl.2; 39-42

124) Aids Group of the United Kingdom Haemophilia Centre Directors with the co-operation of the United Kingdom Haemophilia Directors
Prevalence of antibody to HIV in haemophiliacs in the United Kingdom: a second survey
Clinical Laboratory Haematology 1988; 10; 187-191

125) J A Kanis, E V McCloskey, M Thavarajah, D Evans, N A T Hamdy,
F E Preston, M Greaves
Calcium metabolism and myeloma and the treatment of hypercalcaemia
Haematological Oncology 1988; 6; 115-117

126) F E Preston
Platelets, red cells and vascular disease
Atherosclerosis 1988; 70; 190-191

127) S J Machin, F E Preston
Guidelines on platelet function testing
Journal of Clinical Pathology 1988; 41; 1322-1330

128) S A Solomon, D W K Cotton, F E Preston, L E Ramsay
Severe disseminated intravascular coagulation associated with massive ventricular mural thrombus following acute myocardial infarction
Postgraduate Medical Journal 1988; 64; 791-795

129) G Dolan, M Greaves, P Cooper, F E Preston
Thrombovascular disease and familial plasminogen deficiency: a report of three kindreds
British Journal of Haematology 1988; 70; 417-421

130) J T Wilde, S Kitchen, S Kinsey, M Greaves, F E Preston
Plasma D-dimer levels and their relationship to serum fibrinogen/fibrin degradation products in hypercoaguable states
British Journal of Haematology 1989; 71; 65-70

131) D A Lane, H Erdjument, A Flynn, V Di Marzo, M Panico, H R Morris, M Greaves, G Dolan, F E Preston
Antithrombin Sheffield: amino acid substitution at the reactive site (Arg393 to His) causing thrombosis
British Journal of Haematology 1989; 71; 91-96

132) P Lawson-Matthew, J Clayton, D Guilland-Cumming, A Yates,
F E Preston, M Greaves, J A Kanis
Vitamin D metabolism in myeloma
British Journal of Haematology; 1989; 73; 57-60

133) C Jackson, J Ball, J Peel, J Lawry, M Greaves, F E Preston
DN 9693: A phosphodiesterase inhibitor with a platelet membrane effect
Thrombosis and Haemostasis 1989; 61(2); 266-269

134) M E Nelson, J F Talbot, F E Preston
Recurrent multiple-branch retinal arteriolar occlusions in a patient with protein C deficiency
Graefe's Archive for Clinical and Experimental Ophthalmology 1989; 227; 443-447

1990

135) J T Wilde, P Cooper, H J Kennedy, D R Triger, F E Preston
Coagulation disturbances following ascites recirculation
Journal of Hepatology 1990; 10; 217-222

136) British Committee for Standards in Haematology Haemostasis and Thrombosis Task Force of the British Society for Haematology: Guidelines on oral anticoagulation: second edition
Journal of Clinical Pathology 1990; 43; 177-183

137) J A Garson, F E Preston, M Makris, P Tuke, C Ring, S M Machin, R S Tedder
Detection by PCR of hepatitis C virus in factor VIII concentrates
Lancet, 1990, 335, 1473

138) J A Garson, P W Tuke, M Makris, M Briggs, S J Machin, F E Preston, R S Tedder
Demonstration of viraemia patterns in haemophiliacs treated with hepatitis-C-virus contaminated factor VIII concentrates
The Lancet, 1990, 336, 1022-1025

139) C R M Hay, Y Laurian, F Verroust, F E Preston, P B A Kernoff
Induction of Immune Tolerance in Patients with Hemophilia A and Inhibitors Treated with Porcine VIIIC by Home Therapy
Blood, 1990, 76, 5, 882-886

140) J A Kanis, E V McCloskey, N O'Rourke, F E Preston, M Greaves, K Eyres, S Vasikaran
Biphosphonates in the management of hypercalcaemia of malignancy
1990

141) R G Malia, S Kitchen, M Greaves, F E Preston
Inhibition of activated protein C and its cofactor protein S by antiphospholipid antibodies
British Journal of Haematology 1990, 76, 101-107

142) M D Boisclair, D A Lane, J T Wilde, H Ireland, F E Preston, F A Ofusu
A comparative evaluation of assays for markers of activated coagulation and/or fibrinolysis: Thrombin-antithrombin complex, D-dimer and fibrinogen/fibrin fragment E antigen
British Journal of Haematology, 1990, 74, 471-479

143) D R Triger, F E Preston
Chronic liver disease in haemophilia
British Journal of Haematology, 1990, 74, 241-245 (Annotation)

144) S A Solomon, I Cartwright, G Pockley, M Greaves, F E Preston, L R Ramsay, P C Waller
A placebo-controlled, double blind study of eicosapentaenoic acid-rich fish oil in patients with stable angina pectoris
Current Medical Research & Opinion, 1990, 12(1), 1-11

145) L Poller, J F Davidson, B T Colvin, T W Barrowcliffe, P B A Kernoff
F E Preston (British Society of Haematology)
Guidelines on oral anticoagulation: Second edition
Journal of Clinical Pathology, 1990, 43, 177-183

146) J Ball, L J Warnock, F E Preston
Rapid assessment of haemophilia A carrier state by non-invasive techniques using the polymerase chain reaction
Journal of Clinical Pathology, 1990, 43, 505-507

147) F E Preston, M Makris, D R Triger, M I Adelman, J C E Underwood
A prospective randomised controlled liver biopsy study of recombinant alpha interferon in chronic non-A, non-B hepatitis in haemophilia
Progress in Clinical and Biological Research, Alan R Liss (New York)
Recent Advances in Hemophilia Care (C K Kasper-Editor), 1990,34,135-136

148) CWI Douglas, PR Brown, FE Preston
Platelet aggregation by oral streptococci
F.E.M.S. Microb.Lett., 1990, 72(1-2), 63-68

1991

149) M Greaves, F E Preston
Clinical and laboratory aspects of thrombophilia
Recent Advances in Blood Coagulation (L Poller-Editor)
Churchill Livingstone, 1991, 119-140

150) A M Jennings, I Ford, S Murdock, M Greaves, F E Preston, J D Ward
The effects of diet and insulin therapy on coagulation factor VII, blood viscosity and platelet release proteins in diabetic patients with secondary sulphonylurea failure
Diabetic Medicine, 1991, 8, 346-353

151) M Greaves, F E Preston
Clinical Annotation: The hypercoaguable state in clinical practice
British Journal of Haematology, 1991, 79, 148-151

152) I Ford, T P Singh, S Kitchen, M Makris, J D Ward, F E Preston
Activation of coagulation in diabetes mellitus in relation to the presence of vascular complications
Diabetic Medicine 1991, 8, 322-329

153) M Makris, F E Preston, D R Triger, J C E Underwood, L Westlake, M Adelman
A randomised controlled trial of recombinant alpha interferon in chronic hepatitis C in haemophilia
Blood 1991, 78, 1672-1677

154) K K Hampton, M Makris, S Kitchen, F E Preston
Potential thrombogenicity of heat treated prothrombin complex concentrates in haemophilia B
Blood Coagulation and Fibrinolysis 1991, 2, 637-641

155) L J Warnock, P Brown, F E Preston
GENERSCH: A computer program for restriction fragment analysis
Blood Coagulation and Fibrinolysis 1991, 2, 207-209

156) M G Semple, C Loveday, F E Preston, R S Tedder
Detection of HIV-I RNA in factor VIII concentrate
AIDS, 1991, 5, 597

157) M D Creagh, I F Roberts, D J Clark, F E Preston
Familial antithrombin III deficiency and mycoplasma pneumoniae pneumonia
Journal Clinical Pathology 1991, 44, 870-871

1992

158) P C Cooper, F E Preston
Euglobulin clot lysis time
ECAT Assay Procedures, 1992

159) P M Mannucci, K Schimpf, TR Abe, L M Aledort, K Anderle, D B Brettler, M W Hilgartner, PBA Kernoff, M Kunschak, C W McMillan, F E Preston, G E Rivard and the International Investigator Group
Low risk of viral infection after administration of vapor-heated factor VIII concentrate
Transfusion, 1992, 32, 134-138

160) S Kitchen, R G Malia, F E Preston
A comparison of methods for the measurement of activated factor VII.
Thrombosis and Haemostasis 1992, 68(3), 301-305

161) I Ford, R A Malik, P G Newrick, F E Preston, J D Ward, M Greaves
Relationships between haemostatic factors and capillary morphology in human diabetic neuropathy
Thrombosis and Haemostasis 1992, 68(6), 628-633

1993

162) I Ford, CWI Douglas, F E Preston, A Lawless, K K Hampton
Mechanisms of platelet aggregation by Streptococcus Sanguis, a causative organism in infective endocarditis
British Journal of Haematology 1993; 84: 95-100

163) Makris M, Garson JA, Ring CJA, Tuke PW, Tedder RS, Preston FE
Hepatitis C viral RNA in clotting factor concentrates and the development of hepatitis in recipients.
Blood 1993; 81: 1898-1902

164) Makris M, Preston FE, Triger DR, Underwood JCE, Westlake L, Adelman MI
Interferon Alfa for chronic hepatitis C in haemophiliacs
Gut 1993; Suppl.1: S121-S123

165) Hampton KK, Preston FE, Lowe GDO, Walker ID, Sampson B
Reduced coagulation activation following infusion of a highly purified factor IX concentrate compared to a prothrombin complex concentrate
British Journal of Haematology 1993; 84: 279-284

166) Wodzinski MA, Bardhan KD, Reilly JT, Cooper P, Preston FE
Reduced tissue type plasminogen activator activity of the gastroduodenal mucosa in peptic ulcer disease
Gut 1993; 34: 1310-1314

167) Members of the Haemostasis and Task Force of the British Society for Haematology
Guidelines on the prevention, investigation, and management of thrombosis associated with pregnancy.
Journal of Clinical Pathology 1993; 46: 489-496

168) Makris M, Preston FE
Chronic hepatitis in haemophilia
Blood Reviews 1993; 7: 243-250

169) Greaves M, Preston FE
The diagnosis and clinical management of thrombophilia
Vascular Medicine Review 1993; 4: 117-131

170) Makris M, Greaves M, Preston FE
The use of clotting factor concentrates in the reversal of warfarin in patients with life threatening bleeds
British Journal of Haematology 1993; 84(2): 16

171) Greaves M, Hampton KK, Douglas CWI, Brown PR, Preston FE
Platelet aggregation by streptococci and poor clinical outcome in subacute bacterial endocarditis (SBE)
Thrombosis and Haemostasis 1993; 69(6): 626

172) Douglas CWI, Heath J, Hampton KK, Preston FE
Identity of viridans streptococci recovered from cases of infective endocarditis.
J Med Microbiology 1993

173) Johnson BF, Thomas G, Wiley KN, Greaves M, Preston FE, Fox M,
Raftery AT
Thromboxane and prostacyclin synthesis in experimental pancreas transplantation: Changes in parenchymal and vascular prostanoids
Transplantation 1993; 56(6) 1447-1453

1994

174) Goodeve AC, Chuansumrit A, Sasanakul W, Isarangkura P, Preston FE and
Peake IR
A comparison of the allelic frequencies of ten DNA polymorphisms associated with factor VIII and factor IX genes in Thai and Western European populations.
Blood Coagulation & Fibrinolysis 1994; 5: 29-35

175) Dolan G, Neal K, Cooper P, Brown P and Preston FE
Protein C, antithrombin III and plasminogen: effect of age, sex and blood group.
British Journal of Haematology 1994; 86: 798-803

176) Kitchen S, Walker ID, Woods TAL, Preston FE on behalf of the Steering Committee of the UK National External Quality Assessment Scheme in Blood Coagulation
Thromboplastin related differences in the determination of international normalised ratio: a cause for concern?
Thrombosis and Haemostasis 1994; 72(3): 426

177) Ford I, Li TC, Cooke ID, Preston FE
Changes in haematological indices, blood viscosity and inhibitors of coagulation during treatment of endometriosis with Danazol
Thrombosis and Haemostasis 1994; 72(2); 218-221

178) Henin C, Makris M, Brown J, Peake IR, Preston FE
Peripheral mononuclear cells of haemophiliacs with chronic liver disease are infected with replicating hepatitis C virus
British Journal of Haematology 1994; 87: 215-217

179) Makris M, Preston FE and Ralph S
Increased soluble IL-2 receptor levels in HCV-infected haemophiliacs: a possible indicator of liver disease severity
British Journal of Haematology 1994; 87: 419-421

180) Thomas DP, Hampton KK, Dasani H, Lee CA, Giangrande PLF, Harman C, Lee ML, Preston FE
A cross-over pharmacokinetic and thrombogenicity study of a prothrombin complex concentrate and a purified factor IX concentrate
British Journal of Haematology 1994; 87: 782-788

181) Cartwright IJ, Hampton KK, Macneil S, Colvin BT, Preston FE
A haemorrhagic platelet disorder associated with altered stimulus-response coupling and abnormal membrane phospholipid composition
British Journal of Haematology 1994; 88: 129-136

182) Cooper PC, Hampton KK, Makris M, Abuzenadah A, Paul B, Preston FE
Further evidence that activated protein C resistance can be misdiagnosed as inherited functional protein S deficiency
British Journal of Haematology 1994; 88: 201-203

183) Beauchamp N J, Daly ME, Hampton KK, Cooper PC, Preston FE
High prevalence of a mutation in the factor V gene within the UK population: relationship to activated protein C resistance and familial thrombosis
British Journal of Haematology 1994; 88: 219-222

184) Kitchen S, Walker ID, Woods TAL, Preston FE
Thromboplastin related differences in the determination of International Normalised Ratio: A cause for concern?
Thrombosis and Haemostasis 1994; 72(3): 426-429

185) Al-Saeed A, Makris M, Malia RG, Preston FE, Greaves M
The development of antiphospholipid antibodies in haemophilia is linked to infection with hepatitis C
British Journal of Haematology 1994; 88: 845-848

186) Lane DA, MacGregor IR, Preston FE, Alston TA, Dambra MN
Neutralization of heparin by Trasylol
Annals of Thoracic Surgery 1994; 58(2); 604-605

187) Johnson BF, Wiley KN, Greaves M, Preston FE, Fox M, Raftery AT
Urinary thromboxane and 6-keto-prostaglandin F1alpha are early markers of acute rejection in experimental pancreas transplantation
Transplantation 1994; 58(1); 18-23

188) Saaad S, Rowley G, Tagliavacca L, Green PM, Gianelli F, Rizza CR, Giangrande P, Kernoff PBA, Lee C, Goldman E, McGraw A, Colvin BT, Lucas G, Layton M,, Mibashan RS, Preston FE, Makris M, Mitchel VE, Lowe GDO et al
First report on UK database of haemophilia B mutations and pedigrees
Thrombosis and Haemostasis 1994; 71(5); 563-570

189) Goodeve AC, Preston FE, Peake IR
Factor VIII gene rearrangements in patients with severe haemophilia A
Lancet 1994; 343(8893); 329-330

1995

190) Shapiro A, Abe T, Aledort LM, Anderle K, Hilgartner MW, Kunschak M, Preston FE, Rivard GE, Schimpf K and the International Factor Study Group
Low risk of viral infection after administration of vapor-heated factor VII concentrate or factor IX complex in first-time recipients of blood products.
Transfusion 1995; 35: 204-208

191) Preston FE, Jarvis LM, Makris M, Philip L, Underwood JCE, Simmonds P.
Heterogeneity of HCV genotypes in Hemophilia: Relationship with Chronic Liver Disease
Blood 1995; 85(5); 1259-1262

192) Colvin BT, Hay CRM, Hill FGH, Preston FE
The incidence of FVIII inhibitors in the United Kingdom, 1990-1993
British Journal of Haematology 1995; 89: 908-910

193) Varadi K, Moritz B, Lang H, Bauer K, Preston FE, Peake IR, Rivard GE, Keil B and Schwarz HP
A chromogenic assay for activated protein C resistance
British Journal of Haematology 1995; 90: 884-891

194) Preston FE, Dusheiko G, Lee CA, Ludlam CA, Giangrande PLF
Guidelines on the diagnosis and management of chronic liver disease in haemophilia
Haemophilia 1995; 1(Suppl.4): 42-44

1996

195) Jennings I, Kitchen S, Woods TAL, Preston FE
Development of a World Federation of Hemophilia External Quality Assessment Scheme: results of a pilot study
Haemophilia 1996; 2: 41-46

196) Jarvis Lisa, Ludlam CA, Ellender JA, Nemes L, Field SP, Song E,
Chuansumrit A, Preston FE, Simmonds P
Investigation of the relative infectivity and pathogenicity of different hepatitis C virus genotypes in hemophiliacs
Blood 1996; 87(7): 3007-3011

197) Hay CRM, Colvin BT, Ludlam CA, Hill FGH, Preston FE
Recommendations for the treatment of factor VIII inhibitors: from the UK Haemophilia Centre Directors' Organisation Inhibitor Working Party
Blood Coagulation & Fibrinolysis 1996; 7: 134-138

198) Kitchen S and Preston FE
The therapeutic range for heparin therapy: Relationship between six activated partial thromboplastin time reagents and two heparin assays.
Thrombosis and Haemostasis 1996; 75(5): 734-739

199) Ford I, Douglas CWI, Heath J, Rees C, Preston FE
Evidence for the involvement of complement proteins in platelet aggregation by Streptococcus Sanguis NCTC 7863
British Journal of Haematology 1996, 94: 729-739

200) Kitchen S, Jennings I, Woods TAL, Walker ID, Preston FE
Two Recombinant Tissue Factor Reagents Compared to Conventional Thromboplastins for Determination of International Normalised Ratio: A Thirty-three-laboratory Collaborative Study
Thrombosis and Haemostasis 1996; 76(3): 372-376

201) Beauchamp NJ, Daly ME, Cooper PC, Makris M, Preston FE, Peake IR
Molecular basis of protein S deficiency in three families also showing independent inheritance of factor V Leiden
Blood 1996; 88(5): 1700-1707

202) Makris M, Preston FE, Rosendaal FR, Underwood JCE, Rice KM, Triger DR†
The natural history of chronic hepatitis C in haemophiliacs
British Journal of Haematology 1996; 94: 746-752

203) Goodeve AC, Tagariello G, Chuansumrit A, Preston FE, Peake IR
A rapid and cost effective method for analysis of dinucleotide repeat polymorphisms in the factor VIII gene
Blood Coagulation and Fibrinolysis 1996: 7: 672-677

204) Preston FE, Rosendaal FR, Walker ID, Briet E, Berntorp E, Conard J, Fontcuberta J, Makris M, Mariani G, Noteboom W, Pabinger I, Legnani C, Scharrer I, Schulman S, van der Meer FJM
Increased fetal loss in women with heritable thrombophilia
The Lancet 1996; 348; 913-916

205) Kitchen S, Jennings I, Woods TAL, Preston FE on behalf of the Steering Committee of the UK National Quality Assessment Scheme for Blood Coagulation
Wide variability in the sensitivity of APTT reagents for monitoring of heparin dosage
Journal of Clinical Pathology 1996; 49: 10-14

206) Plater ME, Ford I, Dent MT, Preston FE, Ward JD
Elevated von Willebrand factor antigen predicts deterioration in diabetic peripheral nerve function
Diabetologia 1996; 39; 336-343

1997

207) Makris M, Greaves M, Phillips WS, Kitchen S, Rosendaal FR, Preston FE
Emergency Oral anticoagulant reversal: The relative efficacy of infusions of fresh frozen plasma and clotting factor concentrate on correction of the coagulopathy.
Thrombosis and Haemostasis 1997; 77(3); 477-480.

208) Richards EM, Makris M, Cooper P, Preston FE
In vivo coagulation activation following infusion of highly purified factor XI concentrate
British Journal of Haematology 1997; 96; 293-297

209) Hay CRM, Ollier W, Pepper L, Cumming A, Keeney S, Goodeve AC, Colvin BT, Hill FGH, Preston FE, Peake IR from the UKHCDO Inhibitor Working Party
HLA Class II Profile: A weak determinant of factor VIII inhibitor development in severe haemophilia A.
Thrombosis and Haemostasis 1997; 77(2); 234-237

210) Phillips WS, Smith J, Greaves M, Preston FE, Channer KS
An evaluation and improvement programme for inpatient anticoagulation control.
Thrombosis and Haemostasis 1997; 77(2); 283-288

211) Hampton KK and Preston FE
ABC of Clinical Haematology: Bleeding disorders, thrombosis and anticoagulation
Clinical Review 1997; 314; 1026-1029

212) Ford I, Douglas CWI, Cox D, Rees DGC, Heath J, Preston FE
The role of immunoglobulin G and fibrinogen in platelet aggregation by Streptococcus Sanguis
British Journal of Haematology 1997; 97; 737-746

213) Jennings I, Kitchen S, Woods TAL, Preston FE, Greaves M (on behalf of the UK National External Quality Assessment Scheme for Blood Coagulation)
Potentially clinically important inaccuracies in testing for the lupus anticoagulant: an analysis of results from three surveys of the UK National External Quality Assessment Scheme (NEQAS) for Blood Coagulation
Thrombosis and Haemostasis 1997; 77(5); 934-937

214) Wright JG, Malia RG, Cooper PC, Thomas P, Preston FE, Serjeant GR
Protein C and Protein S in homozygous sickle cell disease: does hepatic dysfunction contribute to low levels?
British Journal of Haematology 1997; 98: 627-631

215) Richards EM, Makris M, Preston FE
The successful use of protein C concentrate during pregnancy in a patient with type 1 protein C deficiency, previous thrombosis and recurrent fetal loss
British Journal of Haematology 1997; 98: 660-661

216) Kitchen S, Preston FE
Monitoring oral anticoagulant treatment with the TAS near-patient test system: comparison with conventional thromboplastins
Journal of Clinical Pathology 1997; 50(11): 951-956

217) Darby SC, Ewart DW, Giangrande PLF, Spooner RJD, Rizza CR, Dusheiko GM, Lee CA, Ludlam CA, Preston FE for the UK Haemophilia Centre Directors' Organisation
Mortality from liver cancer and liver disease in haemophilic men and boys in UK given blood products contaminated with hepatitis C
The Lancet 1997; 350: 1425-1431

218) Makris M, Preston FE, Beauchamp NJ, Cooper PC, Daly ME, Hampton KK, Bayliss P, Peake IR, Miller GJ, "Co-inheritance of the 20210A allele of the prothrombin gene increases the risk of thrombosis in subjects with familial thrombophilia."
Thrombosis and Haemostasis 1997, 78: 1426-1429

1998

219) Perry DJ, Daly ME, Tait RC, Walker ID, Brown K, Beauchamp NJ, Preston FE
Gyde H, Harper PL, Carrell RW
Antithrombin Cambridge II (Ala384Ser): Clinical, Functional and Haplotype Analysis of 18 Families
Thrombosis and Haemostasis 1998; 79: 249-253

220) Beauchamp NJ, Daly ME, Makris M, Preston FE, Peake IR
A novel mutation in Intron K of the PROS1 Gene causes aberrant RNA splicing and is a common cause of Protein S deficiency in a UK thrombophilia cohort
Thrombosis and Haemostasis 1998; 79: 1086-1091

221) Leach M, Makris M, Hampton KK, Preston FE
Norethisterone therapy for bleeding due to gastrointestinal telangiectases in Glanzmann's thrombasthenia (Short report)
British Journal of Haematology 1998: 100: 594-596

222) I Jennings, S Kitchen, TAL Woods, FE Preston
Laboratory performance of haemophilia centres in developing countries: 3 years' experience of the World Federation of Hemophilia External Quality Assessment Scheme
Haemophilia 1998: 4: 739-746

223) Rosendaal FR, Doggen CJM, Zivelin A, Arruda VR, Aiach M, Siscovick DS, Hillarp A, Watzke HH, Bernard F, Cumming AM, Preston FE, Reitsma PH
Geographic Distribution of the 20210G to a Prothrombin Variant
Thrombosis and Haemostasis 1998: 79: 706-708

224) Hay CRM, Ludlam CA, Colvin BT, Hill FGH, Preston FE, Wasseem N,
Bagnall R, Peake IR on behalf of the UK Haemophilia Centre Directors Organisation and also Berntorp E, Mauser-Bunschoten EP, Fijnvandraat K, Kasper CK, White G, Santagostino E.
Factor VIII inhibitors in mild and moderate-severity haemophilia A
Thrombosis & Haemostasis 1998; 79: 762-766

225) Williams IJ, Abuzenadah A, Winship PR, Preston FE, Dolan G, Wright J, Peake IR, Goodeve AC
Precise carrier diagnosis in families with haemophilia A: use of conformation sensitive gel electrophoresis for mutation screening and polymorphism analysis
Thrombosis & Haemostasis 1998; 79: 723-726

226) Beauchamp NJ, Pyke R, Daly M, Butler L, Makris M, Dafforn T, Zhou A, Fitton HL,
Preston FE, Peake IR, Carrell RW
Antithrombin Wibble and Wobble (T85 m/K). Archetypal conformational diseases with in vivo latent-transition, thrombosis and heparin activation
Blood 1998;92:2696-2706

227) Leach M, Makris M, Preston FE
Acute liver failure induced by alcohol and paracetamol in haemophilia
British Journal of Haematology 1998; 103: 891-893

228) Lawrie AS, Kitchen S, Purdy G, Mackie IJ, Preston FE, Machin SJ
Assessment of Actin FS and Actin FSL sensitivity to specific clotting factor deficiencies
Clinical Laboratory Medicine 1998; 20: 179-186

229) Preston FE
Laboratory Diagnosis of hereditary bleeding disorders: External quality assessment.
Haemophilia 1998: 4(Suppl.2) 12-18

230) Kitchen S, Iampietro R, Woolley AM, Preston FE
Anti Xa monitoring during treatment of LMWH of Danaparoid: Inter-Assay variability
Thrombosis and Haemostasis 1998: (Submitted)

231) Preston FE, Kitchen S
Quality control and factor VIII assays:
WFH State-of-the-Art Volume, Haemophilia 1998: 4: 651-653

1999

232) Hinks JL, Winship PR, Makris M, Preston FE, Peake IR, Goodeve AC
A rapid method for haemophilia B mutation detection using conformation sensitive gel electrophoresis (Short report)
British Journal of Haematology 1999: 104: 915-918

233) Kitchen S and Preston FE
Standardization of prothrombin time for laboratory control of oral anticoagulant therapy
Seminars in Thrombosis and Haemostasis 1999: 25(1): 17-25

234) Kitchen S, Iampietro R, Wooley AM, Preston FE
Anti-Xa monitoring during treatment with low molecular weight heparin or Danaparoid: Inter-assay variability
Thrombosis and Haemostasis 1999: 82: 1289-1293

235) Leach M, Makris M, Hampton KK and Preston FE
Spinal epidural haematoma in haemophilia A with inhibitors – efficacy of recombinant factor VIIa concentrate
Haemophilia 1999: 5: 209-212

236) Kitchen S, Cartwright I, Woods, TAL, Jennings I and Preston FE
Lipid composition of seven APTT reagents in relation to heparin sensitivity
British Journal of Haematology 1999: 106: 801-808

237) Kitchen S, Jennings I, Woods TAL, Preston FE
Local calibration of INR improves between laboratory agreement: Results from the UK NEQAS.
Thrombosis and Haemostasis 1999: 81: 60-65

238) I Jennings, S Kitchen, TAL Woods, F E Preston
Normalization does not improve between laboratory agreement but may improve specificity of some assays for activated protein C resistance
Blood Coagulation & Fibrinolysis 1999: 10: 451-453

239) Morsdorf S, Agnelli G, Preston FE, Wenzel E
Optimizing therapy with anticoagulants: risk analysis and socio-economic perspectives. Preface.
Seminars In Thrombosis & Hemostasis; 1999; 25(1): 1-3

24)) Mainwaring C, Makris M, Thomas WE, Hampton KK, Preston FE
Mesenteric infarction due to combined protein C deficiency and prothrombin 20210 defects.
Postgraduate Med.Journal 1999; Dec., 75 (890): 742-743

241) Preston FE, Kitchen S, Jennings I, Woods TAL
A UK National External Quality Assessment Scheme (UK NEQAS) for molecular genetic testing for the diagnosis of familial thrombophilia.
Thrombosis Haemostasis 1999; Nov., 82(5): 1556-1557

242) Nesbitt IM, Hampton KK, Preston FE, Peake IR, Goodeve AC
A common splice site mutation is shared by two families with different type 2N von Willebrand disease mutations.
Thrombosis Haemostasis 1999; Sep., 82(3): 1061-1064

2000

243) Jennings I, Kitchen S, Cooper PC, Rimmer JE, Woods TA, Preston FE
Further evidence that activated protein C resistance affects protein C coagulant activity assays.
Thrombosis Haemostasis 2000; Jan.83(1) 171-172

244) Makris M, Leach M, Beauchamp NJ, Daly ME, Cooper PC, Hampton KK, Bayliss P, Peake IR, Miller GJ, Preston FE
Genetic analysis, phenotypic diagnosis and risk of venous thrombosis in families with inherited deficiencies of protein S.
Blood 2000; Mar.15; 95(6): 1935-1941

245) Kitchen S, Theaker J, Preston FE
Monitoring unfractionated heparin therapy, relationship between eight anti-Xa assays and a protamine titration assay.
Blood Coagulation & Fibrinolysis 2000; Mar.11(2): 137-144

246) Beauchamp NJ, Makris M, Preston FE, Peake IR, Daly ME
Major structural defects in the antithrombin gene in four families with type I antithrombin deficiency partial/complete deletions and rearrangement of the antithrombin gene.
Thrombosis & Haemostasis 2000; May; 83(5) 715-721

247) Visanji JM, Seargent J, Tahri D, Croft SA, Makris M, Preston FE, Peake IR, Daly ME
Influence of the –675 4G/5G dimorphism of the plasminogen activator inhibitor 1 promoter on thrombotic risk in patients with factor V Leiden
British Journal of Haematology 2000; Jul; 110(1): 135-138

248) Hough RE, Hampton KK, Preston FE, Channer KS, West J, Makris M
Recombinant VIIa concentrate in the management of bleeding following prothrombin complex concentrate related myocardial infarction in patients with haemophilia and inhibitors.
British Journal of Haematology 2000; Dec; 111 (3): 974-979

249) Rimmer JE, Cooper PC, Brookfield CJ, Preston FE, Makris M
Evaluation of a global screening assay for the investigation of the protein C
Anticoagulant pathway
Clin.Lab.Haematology 2000, 22(6), 351-354

2001

250) Makris M, Hampton KK, Preston FE
Failure of recombinant FV11a as treatment for abdominal bleeding in acquired hemophilia.American Journal of Hematology 2001, 66: 67-68

251) E S Morris, K K Hampton, I M Nesbitt, F E Preston, W E G Thomas, M MakrisThe management of von Willebrand's disease-associated gastrointestinal angiodysplasia
Blood Coagulation & Fibrinolysis 2001, 12: 143-148

252) Watson HG, Baglin T, Laidlaw SL, Makris M, Preston FE
A comparison of the efficacy and rate of response to oral and intravenous Vitamin K in reversal of over-anticoagulation with warfarin.
British Journal of Haematology 2001, 115(1), 145-149

253) Makris M, Baglin T, Dusheiko G, Giangrande PL, Lee CA, Ludlam CA, Preston FE, Watson HG, Wilde JT, Winter M.
Guidelines on the diagnosis, management and prevention of hepatitis in haemophilia
Haemophilia 2001, 7(4), 339-345

254) Greaves M, Preston FE
Rebuttal to: oral contraceptives and venous thromboembolism
Thrombosis Haemostasis 2001, 85(5), 932-934

255) Morris ES, Hampton KK, Nesbitt IM, Preston FE, Thomas EG, Makris M
The management of von Willebrand's disease-associated gastrointestinal Angiodysplasia
Blood Coagulation & Fibrinolysis 2001, 12(2), 143-148

256) Jennings I, Kitchen S, Smith J, Woods TA, Preston FE
Between-centre agreement in homocysteine assays: experience from the UK NEQAS Proficiency testing scheme.
Thrombosis Haemostasis 2002, 87(5), 921-922

257) Barrowcliffe TW, Mertens K, Preston FE, Ingerslev J
Laboratory aspects of haemophilia therapy.
Haemophilia 2002, 8(3), 244-249

258) Preston FE, Laidlaw ST, Sampson B, Kitchen S
Rapid reversal of oral anticoagulation with warfarin by a prothrombin complex Concentrate (Beriplex): efficacy and safety in 42 patients.
British Journal of Haematology 2002, 116(3), 619-624

MINOR PUBLICATIONS AND ABSTRACTS

1) F E Preston, R G Malia, M J Sworn, E K Blackburn
Intravascular coagulation and renal failure in E.Coli.septicaemia
Journal of Clinical Pathology 1972; 25; 1006

2) A Milford Ward, F E Preston
Fibrin in the kidney in myelomatosis
Journal of Clinical Pathology 1972; 25; 556

3) F E Preston, R G Malia, S E Sherriff, E K Blackburn
Intravascular coagulation and the adult haemolytic-uraemic syndrome.
IVth International Congress on Thrombosis and Haemostasis, Vienna 1973; 441

4) W R Timperley, F E Preston, J D Ward
Intravascular coagulation in the nervous system
VIIth International Congress of Neuropathology, Budapest
Akademai Kiado 1974; 302

5) F E Preston
Essential thrombocythaemia and peripheral vascular disease
British Journal of Haematology 1974; 27; 263

6) W R Timperley, J D Ward, F E Preston, T Duckworth, B C O'Malley
Clinical and histological studies in diabetic neuropathy: Reassessment of the importance of small vessel disease
Neuropathology and Applied Neurobiology 1975; 1; 401

7) W R Timperley, J D Ward, F E Preston, T Duckworth, B C O'Malley
A Reassessment of small vessel disease in diabetic neuropathy
Diabetologia 1975; 11; 367

8) W R Timperley, F E Preston
Intravascular coagulation in the nervous system
Journal of Clinical Pathology 1975; 28; 753

9) W R Timperley, F E Preston, J D Ward
Intravascular coagulation in diabetic keto-acidotic coma
Neuropatholy and Experimental Neurobiology 1975; 1; 107

10) F E Preston, D A Winfield
Prothrombin complex concentrates in liver disease
The Lancet 1975; ii; 328

11) F P Ryan, F E Preston, R C Smart, C D Holdsworth
Abnormalities of splenic function in ulcerative colitis and Crohn's disease
Gut 1975; 10; 834

12) F E Preston, W R Timperley, B C O'Malley, J D Ward
Intravascular thrombosis and platelet function in diabetic neuropathy
Thrombosis Diathesis Haemorrhagic 1975; 34; 891

13) F E Preston, R B Cooke, D A Winfield, D Lee, E K Blackburn
The hyperviscosity syndrome and myelomatosis
International Society of Haematology, European and African Division;
Third Meeting; London; 1975; 41; 18

14) B C O'Malley, F E Preston, W R Timperley, J D Ward
Abnormal platelet function and diabetic neuropathy
Diabetologia; 1975; 11; 367

15) F P Ryan, F E Preston, W R Timperley, C D Holdsworth
Disseminated intravascular coagulation and intestinal disease
Journal of Clinical Pathology; 1976; 29; 1048

16) S M Pritchard, J Watkins, F E Preston, G Hudson
Metabolism of immunoglobulins in vivo and in vitro at various clinical stages of multiple myeloma.
Proceedings VIth European Pathological Symposium; 1976

17) F E Preston, R B Cooke, N F Burrows
Whole blood viscosity studies in myelomatosis
Journal of Clinical Pathology; 1976; 29; 1047-1048

18) F E Preston, R J Sokol, J S Lilleyman, E K Blackburn
Hyperviscosity syndrome in leukaemia
British Journal of Haematology; 1977; 36; 438

19) R G Malia, F E Preston, E K Blackburn
The effect of vitamin K on PIVKA and its associated procoagulant proteins in liver disease
Thrombosis Haemostasis; 1977; 38; 157

20) L C Best, R G G Russell, F E Preston, T J Martin
Responsiveness of adenylate cyclase in human blood platelets
Journal of Endocrinology; 1977; 73; 3

21) F E Preston, C D Holdsworth, R G Malia
Protein induced by vitamin K absence (PIVKA) in liver disease: Response to IV vitamin K therapy
Gut; 1977; 18; 955

22) R G Malia, F E Preston
The influence of PIA II on thrombin generation in liver disease
Thrombosis and Haemostasis; 1977; 38; 288

23) F E Preston, R G Malia, B Sampson
The factor VIII molecule in acquired von Willebrand's disease
Thrombosis and Haemostasis; 1977; 38; 41

24) F E Preston
Circulating platelet aggregates in transient cerebral ischaemia
British Journal of Haematology; 1977; 35; 679

25) J F Martin, J Richmond, V James, F E Preston, J S Lilleyman
Early effects of vinchristine on platelet aggregation
Proceedings of International Society of Haematology; Istanbul;
1977; 412

26) L C Best, R G G Russell, M B McGuire, F E Preston
Normal platelet cyclic nucleotide responses in Glanzmann's thrombasthenia
XVII Congress International Society of Haematology; 1978; 261

27) M B McGuire, R G G Russell, T J Martin, J D Ward, L C Best, F E Preston
Enhanced biosynthesis of malonaldehyde by diabetic platelets
Clinical Science Mol.Medicine; 1978; 54; 1P

28) V E Mitchell, F E Preston, R G Malia
Evidence against vitamin-K deficiency in normal neonates
British Journal of Haematology; 1978; 40; 173-174

29) D A Lane, F E Preston, M E Van Ross, V V Kakkar
FDP in disseminated intravascular coagulation
British Journal of Haematology; 1978

30) R G Malia, F E Preston, V E Mitchell
A PIVKA-like inhibitor in normal neonates
Proceedings XVII International Society of Haematology; 1978; 123

31) P K Bramah, F E Preston
Heterogeneity of platelet release defect in DIC
XVII Congress of International Society of Haematology; 1978; 352

32) L C Best, M B McGuire, T J Martin, F E Preston
The interaction of prostacyclin with compounds which affect platelet function
XVII Congress International Society of Haematology; 1978; 53

33) D R Triger, F E Preston, V E Mitchell, J C E Underwood
Liver biopsy in haemophilia
Gut; 1978; 19; 454

34) F E Preston, B M Sampson, V E Mitchell, N R Porter, R G Malia
The factor VIII response to intravenous DDAVP
XVII Congress of International Society of Haematology; 1978; 83

35) F E Preston, V E Mitchell, D R Triger, J C E Underwood, R M Stewart, G Bardhan, E K Blackburn
Liver biopsy in haemophilia
British Journal of Haematology; 1978; 40; 172

36) M B McGuire, J D Ward, R G G Russell, L C Best, F E Preston
Increased malonyldialdehyde production in vitro by platelets from patients with diabetes mellitus
XVII Congress of International Society of Haematology; 1978; 53

37) L C Best, T K Holland, M B McGuire, J H Scarpello, J D Ward, F E Preston
Mode of action of Dipyridamole on human platelets. Potentiation of prostaglandin effects
Clinical Science Mol. Medicine; 1978; 55; 28

38) L C Best, M B McGuire, F E Preston, R G G Russell
Evidence for a functional ADP receptor in platelets in Glanzmann's thrombasthenia
Clinical Science Mol. Medicine; 1978; 55; 27

39) N R Porter, R G Malia, P C Cooper, F E Preston
The heterogeneity of congenital factor X deficiency
Thrombosis and Haemostasis; 1979; 42; 1; 127

40) J H B Scarpello, L C Best, B H Marcola, M B McGuire, F E Preston, R G G Russell, J D Ward
Platelet function studies in diabetics with peripheral neuropathy
Thrombosis and Haemostasis; 1979; 42; 1; 382

41) R J Davies, D A Lane, M Van Ross, V V Kakkar, F E Preston
Congenital hypofibrinogenaemia in non-identical twins
Thrombosis and Haemostasis; 1979; 42; 1; 934

42) L C Best, P B B Jones, M B McGuire, J B Smith, F E Preston, R G G Russell
Factors influencing thromboxane B2 and malondialdehyde production by human blood platelets
British Journal of Haematology; 1979; 43; 499-500

43) L C Best, M B McGuire, J D Ward, T K Holland, F E Preston, D S Segal, R G G Russell
Effects of Dipyridamole and aspirin on platelet cAMP levels and prostaglandin biosynthesis in vivo in patients with diabetes mellitus
Thrombosis and Haemostasis; 1979; 41; 1; 196

44) S M Whipps, J Watkins, F E Preston, M Eldridge, G Hudson
Disease activity and IgG turnover in IgG myeloma patients
Journal of Clinical Pathology; 1979; 32; 968

45) P C Cooper, F E Preston, R G Malia, L C Best
The effect of leukaemic white cell extracts on platelet aggregation and prostaglandin biosynthesis
Thrombosis and Haemostasis; 1979; 42; 1; 140

46) W Morris-Jones, F E Preston
The dead-digit palpable pulse syndrome. A preliminary study of the value of antiplatelet drugs in this disorder.
Thrombosis and Haemostasis; 1979; 42; 1; 138

47) F E Preston, S Whipps, C A Jackson
Inhibition and recovery of prostacyclin and platelet thromboxane A2 after low dose aspirin.
Proceedings of VIth International Congress on Thrombosis of the Mediterranean League against Thromboembolic Diseases; Monte Carlo; 1980; 252

48) C A Jackson, F E Preston
The relationship between platelet aggregation responses and thromboxane production following aspirin (ASA).
Proceedings of VIth International Congress on Thrombosis of the Mediterranean League Against Thromboembolic Diseases; Monte Carlo; 1980; 309

49) C A Jackson, A Patterson, C B Brown, F E Preston
Spontaneous platelet aggregation and increased platelet thromboxane production in nephrotic syndrome. Relationship to albumin.
Proceedings of VIth International Congress on Thrombosis of the Mediterranean League Against Thromboembolic Diseases; Monte Carlo; 1980; 148

50) W Morris-Jones, F E Preston, M Greaney, D K Chatterjee
Aspirin and Dipyridamole in peripheral gangrene
Acta Therapeutica; 1980; 6; 94

51) F E Preston
Inhibition of prostacyclin and platelet thromboxane A2 by aspirin
New England Journal of Medicine; 1981; 304; 1174-1175

52) J Watkins, G Wild, F E Preston, V Boyd, T J Hughes, C V Levy
Haematological and immunological sequelae of anaesthetic and surgical trauma
British Journal of Anaesthesia; 1981; 52; 49

53) A D Paterson, D J Beard, D L Douglas, J A Kanis, F Neale, F E Preston, R G G Russell
Diphosphonates are effective in controlling hypercalcaemia in malignant disease
Clinical Science; 1981; 62; 41

54) C A Jackson, F E Preston, M Greaves
Dipyridamole reverses the inhibitory effect of aspirin on prostacyclin synthesis
Thrombosis and Haemostasis; 1981; 46; 26

55) P R Heath, R G Malia, S Tomlinson, F E Preston
The use of a cell-free synthesis system for the study of factor VIII
Thrombosis and Haemostasis; 1981; 46; 166

56) N R Porter, P R Brown, R G Malia, F E Preston
Measurement of platelet nucleotides by reverse-phase HPLC and the demonstration of acquired storage pool defect in DIC
Thrombosis and Haemostasis; 1981; 46; 415

57) M Greaves, F E Preston
The fibrinolytic response to stanozolol in normal subjects
Thrombosis and Haemostasis; 1981; 46; 388

58) C A Jackson, F E Preston, M Greaves
Dipyridamole reverses the inhibitory effect of aspirin on prostaglandin synthesis
Thrombosis and Haemostasis; 1981; 46; 26

59) W Morris-Jones, F E Preston
Non-Surgical management of peripheral vascular disease
British Medical Journal; 1981; i; 317

60) P R Heath, R G Malia, S Tomlinson, F E Preston
The use of a cell-free synthesis system for the study of factor VIII
Thrombosis and Haemostasis; 1981; 46; 166

61) F E Preston, C A Jackson, M Greaves, C J Stoddard
Inhibition and recovery of prostacyclin and thromboxane A2 after low-dose aspirin
Thrombosis and Haemostasis; 1981; 46; 25

62) M Greaves, F E Preston
The fibrinolytic response to stanozolol in normal subjects
Thrombosis and Haemostasis; 1981; 46; 388

63) F E Preston
The role of platelets in thrombotic disorders
British Journal of Haematology; 1981; 49; 143-144

64) F E Preston; R G Malia
The factor VIII response to high purity porcine factor VIII concentrate in acquired von Willebrand's disease
Thrombosis and Haemostasis; 1981; 46; 336

65) N R Porter, P R Brown, R G Malia, F E Preston
Measurement of platelet nucleotides by reverse-phase HPLC and the demonstration of acquired storage pool defect in DIC
Thrombosis and Haemostasis; 1981; 46; 415

66) C Kluft, F E Preston, R G Malia, G Wijngaards
haracterization of the effect of stanozolol on fibrinolytic parameters in healthy olunteers
Haemostasis; 1982; 11; Suppl.1; 98

67) F E Preston, R G Malia, M Greaves, G Wijngaards, C Kluft
The effect of stanozolol on thrombotic risk factors in healthy individuals
Haemostasis; 1982; 11; Suppl.1; 99

68) R M Bertina, F E Preston, R G Malia, S L Blamey, G D O Lowe, C D Forbes
Protein C is increased in healthy volunteers and surgical patients after treatment with stanozolol
Thrombosis and Haemostasis; 1983; 50; 349

69) A E Bloom, M Greaves, A J Nicholls, F E Preston, C B Brown
Reduced platelet sensitivity to prostacyclin (PGI2) in focal and segmental glomerulosclerosis (FSGS)
Thrombosis and Haemostasis; 1983; 50; 392

70) V Sawhney, M Greaves, F E Preston
The immediate effects of eicosapentaenoic acid on platelet reactivity in man
Thrombosis and Haemostasis; 1983; 50; 280

71) B E Woodcock, P C Cooper, P R Brown, C Pickering, D A Winfield
F E Preston
The platelet defect in acute myeloblastic leukaemia
Thrombosis and Haemostasis; 1983; 50; 360

72) J Galloway, B E Woodcock, V Sawhney, M Greaves, R G G Russell,
F E Preston
A study of the fatty acid composition of platelet membrane phospholipids after dietary supplementation with a fish oil; failure of incorporation of eicosapentaenoic acid into phosphatidylinositol
Thrombosis and Haemostasis; 1983; 50; 363

73) F E Preston
Aspirin prostaglandins and peripheral gangrene
American Journal of Medicine; 1983; 74; 55-60

74) J Galloway, B E Woodcock, V Sawhney, M Greaves, R G G Russell,
F E Preston
The effect of fish oil on platelet function is not explained by release of eicosapentaenoic acid EPA from the platelet membrane phospholipid pool
British Journal of Haematology; 1983; 55; 171

75) R G Malia, J Ball, T Durrant, S Middleton, F E Preston
The characterisation of the factor in porcine plasma which aggregates human platelets
Thrombosis and Haemostasis; 1983; 50; 210

76) Gugliotta, C Pickering, M Greaves, F E Preston
Abnormality of platelet membrane glycoproteins in essential thrombocythaemia
Thrombosis and Haemostasis; 1983; 50; 216

77) C Pickering, M Greaves, F E Preston, A J M Boulton, J D Ward
The influence of blood glucose control on platelet membrane glycoproteins in insulin-dependent diabetic subjects
Thrombosis and Haemostasis; 1983; 50: 390

78) R G Malia, S Middleton, J Ball, T Durrant, F E Preston
The interaction between native and purified porcine plasma and human platelets
Thrombosis Haemostasis; 1983; 50; 449

79) J M Seid, R G Malia, B Sampson, J Ball, F E Preston, S Tomlinson
Biosynthesis of factor VIII related antigen (VIIRag) by porcine aortic endothelial cells in culture
Clinical Science; 1983; 64; 83

80) A E Bloom, M Greaves, A J Nicholls, F E Preston, C B Brown
Reduced platelet sensitivity to prostacyclin (PGI2) in focal and segmental glomerulosclerosis (FSGS)
Proceedings of the XXth Congress of the European Dialysis and Transplant Association; 1983

81) M Greaves, C Pickering, N R Porter, J M Magee, F E Preston
Acquired Glanzmann's thrombasthenia
Blood; 1983; 61; 209

82) F E Preston, L C Best, M Greaves, C Pickering
A mild bleeding tendency associated with abnormal platelet membrane phospholipid turnover; a case of phospholipase deficiency?
Thrombosis and Haemostasis; 1983; 50; 363

83) F E Preston, B E Woodcock, J Galloway, M Greaves, V Sawhney
The effects of eicosapentaenoic acid supplementation on platelets in patients with intermittent claudication: a double-blind study
Thrombosis and Haemostasis; 1983; 50; 335

84) M Greaves, F E Preston, J D Ward
Changes in plasma plasminogen activator and fibrinogen concentrations in response to improved glycaemic control in diabetic subjects
Symposium on Clinical Aspects of Fibrinolysis and Thrombolysis
Denmark; September 1983

85) C Kluft, J H Verheijen, P Cooper, G T G Chang, A F H Jie, S L Blamey, G D O Lowe, C D Forbes, F E Preston
Post-operative changes in the activity in blood of extrinsic (tissue-type) plasminogen activator and its fast-acting inhibitor
Haemostasis; 1984; 41; 26

86) J H Verheijen, D C Rijken, G T G Chang, F E Preston, C Kluft
The level of rapid tissue-type plasminogen activator inhibition in plasma is decreased by oral administration of stanozolol
Haemostasis; 1984; 50; 31

87) F E Preston, C Kluft, M Simms, R G Malia, M Greaves
Comparison of effects of stanozolol and sub-cutaneous heparin in coagulation and fibrinolysis in surgical patients
Haemostasis; 1984; 51; 31

88) J Ball, R G Malia, M Greaves, F E Preston
Assessment of factor VIII multimers in four cases of acquired von Willebrand's disease associated with a circulating inhibitor to the factor VIII complex
Thrombosis and Haemostasis; 1985; 54; 191

89) R G Malia, H J Kennedy, B K Park, D R Triger, F E Preston
The effect of acetaminophen on the vitamin K dependent prothrombin complex
Thrombosis and Haemostasis; 1985; 54; 205

90) C R M Hay, F E Preston, D R Triger, J C E Underwood, M Greaves, J S Lilleyman
Progressive chronic liver disease in haemophilia
Thrombosis and Haemostasis; 1985; 54; 20

91) D R Triger, F E Preston, R G Malia
Coagulation and platelet function in patients with impaired liver function receiving cefotetan. Cefotetan - a long acting antibiotic for polymicrobila infections
Churchill Livingstone; 1985; 143-146

92) P C Cooper, R D E Battersby, F E Preston, M Greaves
The fibrinolytic activity of chronic subdural haematoma fluid and capsule tissue
British Society for Haemostasis and Thrombosis

93) C Pickering, I J Cartwright, J Martin, F E Preston, M Greaves
A new familial giant platelet syndrome with structural, metabolic and functional abnormalities of blood platelets due to a primary megakaryocyte defect
British Journal of Haematology; 1986; 63; 199-200

94) M Greaves, I J Cartwright, A G Pockley, J H Galloway, B E Woodcock F E Preston
Fish consumption and mortality from coronary heart disease
New England Journal of Medicine; 1985; 3; 822

95) C P Gibbons, K N Wiley, A T Raftery, M Greaves, F E Preston
Prostaglandin synthesis in experimental renal allograft rejection
British Journal of Haematology; 1986; 63; 200

96) P B A Kernoff, E J Miller, G F Savidge, S J Machin, M S Dewar, F E Preston
Reduced risk of Non-A, Non-B Hepatitis after a first exposure to wet-heated factor VIII concentrate
British Journal of Haematology; 1986; 64; 827

97) C R M Hay, F E Preston, D R Triger, J C E Underwood
Liver disease in haemophilia
The Lancet; 1985; 2; 1187

98) C Pickering, J Ball, F E Preston, A J Boulton, J D Ward, G Knight M Greaves
The factor VIII complex in diabetic ketoacidosis
Diabetes Research and Clinical Practice; 1985; 1; 1; S447

99) M Greaves, C Pickering, I Gugliotta, F E Preston
Platelet membrane glycoprotein abnormalities in myeloproliferative disease
British Journal of Haematology; 1986; 6; 4; 780-782

100) P C Waller, C R M Hay, H A Cameron, C Carter, L Parnell, M Greaves, F E Preston, L E Ramsay
Placebo controlled trial of Iloprost in patients with stable intermittent claudication
British Journal of Clinical Pharmacology; 1986; 21; 562-563

101) A Bloom, M Greaves, F E Preston, C B Brown
Evidence against a platelet cyclo-oxygenase defect in uraemic subjects on chronic haemodialysis
British Journal of Haematology; 1986; 62; 143-149

102) J Wright, P Cooper, F E Preston, M Greaves
Changes in the plasma concentration of tissue plasminogen activator and its inhibitors during normal human pregnancy
British Journal of Haematology; 1986;

103) F E Preston
Disseminated intravascular coagulation: New approaches to diagnosis and treatment
Thrombosis Research; 1986; Suppl.IV; 12

104) I J Cartwright, C Pickering, J Galloway, F E Preston, M Greaves
Relationship between fatty acid composition of platelet membrane phospholipid and prostaglandin biosynthesis in diabetes mellitus
Thrombosis Research; 1986; Suppl.IV; 3

105) F E Preston, C R M Hay, D R Triger, M Greaves, J C E Underwood
Predictive markers of chronic liver disease in haemophilia
La Ricerca in Clinica E in Laboratori Italian Version; 1986;
Vol XVI; 147

106) M S Dewar, J Dawes, D A Pratt, F E Preston
Direct evidence that plasma thrombospondin is not a platelet-specific marker
Thrombosis and Haemostasis: 1986;

107) H J Kennedy, P C Cooper, L Creighton, P J Gaffney, F E Preston, D R Triger
The effect of ascites reinfusion and coagulation
Journal of Hepatology; 1986; 3; 6

108) S Kitchen, R G Malia, M Greaves, F E Preston
Comparison of human and rabbit brain thromboplastin in evaluation of haemostatic defect of liver disease
The Lancet; 1986; II; 1463

109) J Ball, R G Malia, M Greaves, F E Preston
Factor VIII multimeric structure in acquired von Willebrand's disease
British Journal of Haematology; 1986; 64; 819

110) R C Collin, M Greaves, F E Preston
Vinchristine/Adriamycin/Dexamethosone combination chemotherapy in refractory myeloma
British Journal of Haematology; 1986; 64; 826

111) J Wright, P C Cooper, F E Preston, M Greaves
Changes in the plasma concentration of tissue plasminogen activator and its inhibitors during normal human pregnancy
British Journal of Haematology; 1987; 66; 424

112) J Ball, M Greaves, C Jackson, J Peel, F E Preston
A phosphodiesterase inhibitor with a platelet membrane effect
Thrombosis and Haemostasis; 1987; 58; 212

113) J Ball, R G Malia, M Greaves, F E Preston
Paradoxical increase in human factor VIII after infusion of porcine factor VIII concentrate in a patient with acquired variant von Willebrand's disease
Thrombosis and Haemostasis; 1987; 58; 361

114) P C Cooper, D R Triger, H Kennedy, R G Malia, F E Preston
Fibrinolysis and haemostasis during ascites recirculation
Thrombosis and Haemostasis; 1987; 58; 76

115) S M Cooper, R G Malia, F E Preston, S L B Duncan, A R B Smith, M Greaves
Clinical and laboratory features associated with lupus anticoagulant
Thrombosis and Haemostasis; 1987; 58; 391

116) R S Evely, F E Preston, D R Triger, C R M Hay, M Greaves, J C E Underwood
Type III pro-collagen peptide in liver disease in haemophilia
Thrombosis and Haemostasis; 1987; 58; 338

117) I Ford, P G Newrick, R A Malik, F E Preston, J D Ward, M Greaves
Haemostatic parameters, endoneurial oxygen tension and sural nerve histology in diabetes mellitus
Thrombosis and Haemostasis; 1987; 58; 88

118) S Kitchen, R G Malia, D R Triger, M Greaves, F E Preston
Comparison of human and rabbit brain thromboplastin in the evaluation of liver disease
Thrombosis and Haemostasis; 1987; 58; 78

119) S Kitchen, R G Malia, M Greaves, F E Preston
Determination of activated factor VII by bovine and rabbit thromboplastins
Thrombosis and Haemostasis; 1987; 58; 134

120) R G Malia, H J Kennedy, D R Triger, F E Preston
Protective effect of vitamin K against acetaminophen (Paracetomol) toxicity in the hamster
Thrombosis and Haemostasis; 1987; 58; 420

121) F E Preston, M Greaves, B Sampson, P B A Kernoff, G Savidge, N Bax
Hypothyroidism and acquired von Willebrand's disease
Thrombosis and Haemostasis; 1987; 58; 362

122) J T Wilde, S Kitchen, F E Preston, M Greaves
A comparison of D-dimer and serum fibrinogen/fibrin degradation product levels (FDP's) in the investigation of hypercoaguable states
Thrombosis and Haemostasis; 1987; 58; 97

123) J T Wilde, H L Smart, D R Triger, D A Lane, F E Preston
Plasma D-dimer and serum fragment E levels in patients with chronic liver disease
Journal of Hepatology; 1987;

124) D R Triger, R G Malia, F E Preston
Platelet function and coagulation in patients with hepatobiliary disorders receiving Cefotetan prophylaxis
Infection 16; 1988; 2; 105-108

125) J T Wilde, D A Lane, E Thompson, W E G Thomas, M Greaves, F E Preston
Acquired dysfibrinogenaemia occurring as a complication of acute pancreatitis
British Journal of Haematology; 1988; 68; 510

126) R G Malia, S M Cooper, F E Preston, M Greaves
The sensitivity of the dilute Russell Viper venom test to the lupus anticoagulant
Clinical and Experimental Rheumatology; 1988; 6; 2

127) R G Malia, S M Cooper, F E Preston, M Greaves
Problems with the standardisation of a routine anticardiolipin assay
Clinical and Experimental Rheumatology; 1988; 6; 2

128) Antithrombin Sheffield: A variant with impaired reactivity towards thrombin
D A Lane, H Erdjument, A M Flynn, M Greaves, F E Preston
British Journal of Haematology, 1988; 69 (1); 141; P65

129) M Greaves, R G Malia, S M Cooper, F E Preston
Antiphospholipid antibody syndrome
Journal of the Royal Society of Medicine 1988, 81, 59

130) J Ball, F E Preston
Non invasive techniques for the assessment of haemophilia A carrier status using the PCR
British Journal of Haematology; 1989; 71; 2; Suppl.1

131) S Kitchen, R G Malia, B M Sampson, M Greaves, F E Preston
Inhibition of protein S co-factor activity by antiphospholipid antibodies
British Journal of Haematology; 1989; 71; 3; Suppl.1.

132) M Makris, F E Preston, D R Triger, M I Adelman, J C E Underwood
A prospective randomised controlled liver biopsy study of recombinant alpha interferon in chronic Non-A, Non-B hepatitis in haemophilia
British Journal of Haematology; 1989; 71; 19; Suppl.1.

133) C R M Hay, P B A Kernoff, F E Preston
Porcine factor VIII for home therapy of haemophiliacs with inhibitors
British Journal of Haematology; 1989; 71; 20; Suppl.1.

134) J T Wilde, P C Cooper, H J Kennedy, D R Triger, F E Preston
An investigation into the coagulation disturbance of ascites recirculation
British Journal of Haematology; 1989; 71; 37; Suppl.1.

135) K K Hampton, I J Cartwright, S MacNeil, B T Colvin, F E Preston
A bleeding disorder associated with altered stimulus-response coupling
Proceedings of the British Society for Haemostasis & Thrombosis
Sheffield, September 1989, 7, 25

136) C R M Hay, P B A Kernoff, F E Preston, Y Laurian, F Verroust
Home therapy of factor VIII inhibitor patients with porcine factor VIII: Evidence for the induction of immune-tolerance
Proceedings of the British Society for Haemostasis & Thrombosis,
Sheffield, September 1989, 8, 29

137) E V McCloskey, M Beneton, S Harris, M Greaves, F E Preston, J A Kanis
Diphosphonates in Multiple Myelomatosis
Classified Tissue International; 1989; 44 (Supplement); S106

138) M Makris, F E Preston, D R Triger, J C E Underwood
A Prospective Randomised Cross-over Study of Recombinant Alpha Interferon in Chronic Hepatitis C in Haemophilia
Gut, 1990, 31, 591

139) E V McCloskey, M Beneton, S Harris, M Greaves, F E Preston, J A Kanis
Diphosphonates in Multiple Myelomatosis
Bone, 1989, 10, 476(P6)

140) M Makris, M S Dewar, S Kitchen, M Greaves, F E Preston
Potential thrombogenicity of clotting factor concentrates
Presented at the British Society for Haemostasis and Thrombosis
Scientific Meeting, London, February 1989 (Abstract Book Pg 18)

1990

141) J A Garson, D Barker, F E Preston, S J Machin, J Barbara,
R S Tedder, et al
Development and Evaluation of "Nested" PCR for the Detection of Hepatitis C Viral RNA in Clinical Samples
Presented VIIIth International Congress of Virology, Berlin 26-31 August 1990

142) M Makris, F E Preston, D R Triger, J C E Underwood, G Kuo, Q-L Choo,
M Houghton
The role of Hepatitis C virus in chronic liver disease in haemophilia.
British Journal of Haematology, 1990, 74, Suppl.1 (Abstract) Page 6

143) S Kitchen, R G Malia, M Greaves, F E Preston
Inhibitory effect of antiphospholipid antibodies on the Protein C/
Protein S pathway: A Possible mechanism of thrombosis
British Journal of Haematology, 1990, 74, Suppl.1 (Abstract) Page 16

144) K K Hampton, B M Sampson, M I Adelman, F E Preston
A new variant of von Willebrand's disease: A hybrid of Type IIb Tampa and platelet-type von Willebrand's disease
British Journal of Haematology, 1990, 74, Suppl.1 (Abstract) Page 16

145) M Makris, M S Dewar, F E Preston, Q-I Choo, G Kuo, M Houghton
The relation of hepatitis C antibodies to acute non-A, non-B hepatitis (NANBH) in previously untreated haemophilia patients
British Journal of Haematology, 1990, 74, Suppl.1 (Abstract) Page 44

146) Montandon AJ, Makris M, Green PM, Coffey AJ, Preston FE, Giannelli F
Clinically moderate to severe haemophilia B in a woman heterozygous for a nonsense mutation of the factor IX gene
British Journal of Haematology, 1990, 76, Suppl.1, 8 (Abstract)

147) Makris M, Kitchen S, Russell FM, Preston FE
Clinical use of activated recombinant factor VII concentrate (rFVIII) and its in vivo monitoring
British Journal of Haematology, 1990, 76, Suppl.1, 9 (Abstract)

148) Makris M, Al-Saeed A, Malia RG, Cooper SM, Greaves M, Preston FE
Anticardiolipin antibodies and lupus anticoagulant in haemophilia; relation to HIV and hepatitis C infection
British Journal of Haematology, 1990, 76, Suppl.1, 15 (Abstract)

149) Cooper PC, Malia RG, Makris M, Preston FE
A new functional assay of Protein S
British Journal of Haematology, 1990, 76, Suppl.1, 22 (Abstract)

150) R G Malia, S Kitchen, M Greaves, F E Preston
The inhibitory effect of antiphospholipid antibodies on the natural anticoagulant Protein C/Protein S pathway
Clinical and Experimental Rheumatology, 1990, 8, 2:208

1991

151) A Al-Saeed, R G Malia, M Makris, S Cooper, F E Preston, M Greaves
The development of antiphospholipid antibodies in haemophilia is strongly linked to infection with hepatitis C
Thrombosis Haemostasis 1991, 65, 1257

152) G Dolan, P Cooper, P Brown, B Sampson, K Forman, F E Preston
Variation in plasma protein C concentrations in healthy volunteers and the significance of low values
British Journal of Haematology 1991, 77, Suppl.1, 10

153) K K Hampton, C Brookfield, R G Malia, F E Preston
The effect of iodinated contrast media on coagulation in-vitro
British Journal of Haematology 1991, 77, Suppl.1, 42

154) F M Russell, C Brookfield, P C Cooper, F E Preston
Maxepa and fibrinolytic activity in healthy volunteers
British Journal of Haematology 1991, 77, Suppl.1 45

155) M A Wodzinski, K D Bardhan, P C Cooper, J T Reilly, F E Preston
Alterations in mucosal-type and urokinase-type plasminogen activator activity in peptic ulcer disease
British Journal of Haematology 1991, 77, Suppl.1. 46

156) G Dolan, P C Cooper, P Jackson, B Sampson, K Forman, F E Preston
Plasma antithrombin III and plasminogen in healthy volunteers
British Journal of Haematology 1991, 77, Suppl.1 60

157) I Ford, T C Li, R G Malia, I D Cooke, F E Preston
Changes in the natural inhibitors of coagulation, ATIII, Protein C and Protein S during treatment with Danazol
British Journal of Haematology 1991, 77, Suppl.1 61

158) K K Hampton, C W I Douglas, P Brown, F E Preston
Platelet aggregation by oral streptococci and bacterial endocarditis
British Journal of Haematology 1991, 77, Suppl.1 78

159) K K Hampton, CWI Douglas, P Brown, FE Preston
Platelet aggregation by oral streptococci and bacterial endocarditis
Thrombosis and Haemostasis 1991; 65: 1032

160) Douglas CWI, Hampton KK, Brown PR, Preston FE
Bacterial endocarditis and platelet aggregation by oral streptococci.
J Dent Research 1991; 70: 680

161) K K Hampton, C Brookfield, R G Malia, F E Preston
The effect of iodinated contrast media on platelets and fibrinolysis in-vitro
British Journal of Haematology 1991, 77, Suppl.1 80

162) I Ford, M T Dent, M Greaves, F E Preston
Interactions between haemostasis and lipids in patients with early Type I diabetes
British Journal of Haematology 1991, 77, Suppl.1 84

163) M A Wodzinski, K D Bardhan, P Cooper, J T Reilly, F E Preston
Mucosal tissue-type and urokinase-type plasminogen activator activity in peptic ulcer disease
Thrombosis Haemostasis 1991, 65, 888

164) M A Wodzinski, K D Bardhan, P Cooper, J T Reilly, F E Preston
Mucosal tissue-type plasminogen activator activity in peptic ulcer disease
Gut, 1991, 35 (Abstract)

165) I Ford, M T Dent, M Greaves, F E Preston, R M Wilson, J D Ward
Interactions between haemostasis and lipids in patients with early Type I diabetes
Thrombosis Haemostasis 1991, 65, Suppl.6, 986

166) R G Malia, S M Cooper, M Hill, F E Preston, M Greaves
Identification of the lupus anticoagulant in patients receiving oral anticoagulants
Thrombosis Haemostasis 1991, 65, Suppl.6 1003

167) Makris, K K Hampton, S Kitchen, M Greaves, F E Preston
Thrombogenicity of prothrombin complex concentrates: Relation to liver disease
Thrombosis Haemostasis 1991, 65, Suppl.6, 1166

168) R G Malia, M Hill, P Cooper, M Greaves, F E Preston
Measurement of protein S in patients with the lupus anticoagulant
Thrombosis Haemostasis 1991, 65, Suppl.6, 1203

169) A Al-Saeed, R G Malia, M Makris, S Cooper, F E Preston, M Greaves
The development of antiphospholipid antibodies (APA) in haemophilia is strongly linked to infection with hepatitis C
Thrombosis Haemostasis 1991, 65, Suppl.6, 1257

170) R G Malia, S M Cooper, M Hill, F E Preston, M Greaves
Identification of the lupus anticoagulant (LA) in the presence of heparin therapy
Thrombosis Haemostasis 1991, 65, Suppl.6, 1317

1992

171) A M Lawless, I Ford, C W I Douglas, F E Preston
Mechanisms of platelet aggregation by oral streptococci
Journal of Dental Research 1992 72: 694

172) Ford I, Douglas CWI, Lawless AM, Hampton KK, Preston FE
Aggregation of platelets by Streptococcus Sanguis requires glycoprotein 1b as well as glycoprotein 11b/111a, and is dependent on the cyclo-oxygenase pathway
British Journal of Haematology 1992; 80 Suppl.1: 6

1993

173) Ford I, Douglas CWI, Shaw J, Hampton KK, Preston FE
The interaction between bacterial cells and a plasma component is an essential step in the platelet aggregation response to Streptococcus Sanguis.
Blood Coagulation and Fibrinolysis 1993; 4: 353

174) Shaw J, Ford I, Douglas CWI, Preston FE
An essential plasma component in platelet aggregation by Streptococcus Sanguis
J Dental Res 1993;

175) Greaves M, Hampton KK, Douglas CWI, Brown PR, Preston FE
Platelet aggregation by streptococci and poor clinical outcome in subacute bacterial endocarditis
Thrombosis Haemostasis 1993; 69(6): 626

176) Makris M, Greaves M, Preston FE
The use of clotting factor concentrates in the reversal of warfarin in patients with life-threatening bleeds
British Journal of Haematology 1993; 84(2): 16

177) Phillips W, Makris M, Preston FE
Audit of the frequency and clinical response to excessive oral anticoagulation
British Journal of Haematology 1993; Suppl.1: 57

178) Ford I, Douglas CWI, Shaw J, Hampton KK, Preston FE
The interaction between bacterial cells and a plasma component is an essential step in the platelet aggregation response to Streptococcus Sanguis.
Thrombosis Haemostasis 1993; 69: 909

1994

179) Cooper PC, Hampton KK, Abuzenadah A, Malia RG, Preston FE, Greaves M
Activated protein C resistance in antiphospholipid containing plasma: A possible mechanism of thrombosis.
British Journal of Haematology 1994; 86(Suppl.1): 2

180) Ford I, Douglas CWI, Preston FE
Streptococcus Sanguis-induced platelet aggregation requires activation of complement.
British Journal of Haematology 1994; 86(Suppl.1): 14

181) Hampton KK, Cooper PC, Abuzenadah A, Preston FE
Resistance to activated Protein C in type II (functional) protein S deficiency
British Journal of Haematology 1994; 86(Suppl.1): 23

182) Cooper PC, Abuzenadah A, Preston FE
APC Resistance test, a new phenomenon - the role of platelets
British Journal of Haematology 1994; 86(Suppl.1): 33

183) Jennings I, Kitchen S, Woods TAL, Preston FE
Measurement of protein S, problems and findings from the National External Quality Assessment Scheme (NEQAS) in Blood Coagulation
British Journal of Haematology 1994; 86(Suppl.1): 32

184) Kitchen S, Woods TAL, Jennings I, Preston FE
An improved method of External Quality Assessment of Heparin dosage
British Journal of Haematology 1994; 86(Suppl.1): 34

185) Douglas CWI, Ford I, Preston FE
The role of complement in the aggregation of platelets by Streptococcus Sanguis
Journal of Dental Research 1994

186) Schwarz HP, Varadi K, Moritz B, Lang H, Bauer K, Preston FE, Peake IR, Rivard GE.
A chromogenic assay for activated protein C (APC)
American Society for Haematology 1994

1995

187) Makris M, Beauchamp N, Cooper P, Daly M, Peake IR, Preston FE
Comparison of the morbidity patterns of homozygous and heterozygous patients with activated protein C resistance
British Journal of Haematology 1995: 89 (Suppl.1) 7:(23)

188) Preston FE, Dusheiko G, Giangrande PLF, Lee CA, Ludlam CA, Darby S on behalf of the UK Haemophilia Centre Directors Organisation.
Hepatocellular carcinoma in UK haemophiliacs
British Journal of Haematology 1995: 89 (Suppl.1) 9:(30)

189) Phillips WS, Collin RC, Preston FE, Makris M, Greaves M
Application of the method of Rosendaal et al to the assessment of anticoagulant control in a number of clinical settings
British Journal of Haematology 1995: 89 (Suppl.1) 28:(103)

190) Makris M, Kitchen S, Phillips WS, Greaves M, Preston FE
The relationship between anti-Xa level and bleeding in over-anticoagulated patients
British Journal of Haematology 1995: 89 (Suppl.1) 29:(105)

191) Phillips WS, Preston FE, Brown JD, Collin RC, Prangnell DR
Over-anticoagulation, risk of haemorrhage and mortality
British Journal of Haematology 1995: 89 (Suppl.1) 29:(106)

192) Makris M, Preston FE
The use of antithrombin III concentrate in pregnant women with familial ATIII deficiency
British Journal of Haematology 1995: 89 (Suppl.1) 30:(108)

193) Jennings I, Woods TAL, Kitchen S, Preston FE for UK NEQAS in Coagulation Sensitivity of activated partial thromboplastin time (APTT) reagents to intrinsic pathway factors - findings of the UK National External Quality Assessment Scheme (NEQAS) for Blood Coagulation
British Journal of Haematology 1995: 89 (Suppl.1) 50:(182)

194) Kitchen S and Preston FE
The ISI/INR system is an inappropriate model for heparin dosage monitoring using the APTT
British Journal of Haematology 1995: 89: (Suppl.1) 50:(183)

195) Kitchen S, Jennings I, Woods TAL, Preston FE
A multicentre comparison of two recombinant thromboplastins and conventional thromboplastins for determination of international normalised ratio
British Journal of Haematology 1995: 89: (Suppl.1) 69:(252)

196) Woods TAL, Jennings I, Kitchen S, Preston FE
Significant differences between methodologies in fibrinogen measurement. Findings of the UK National External Quality Assessment Scheme (UK NEQAS)
British Journal of Haematology 1995: 89: (Suppl.1) 69:(253)

1996

197) Hay CRM, Ludlam CA, Colvin BT, Hill FGH, Preston FE, Mauser-Bunschoten EP, Finvandraat K, Sultan Y, White G, Kasper C, UKHCDO Inhibitor Working Party c/o University of Manchester
Factor VIII inhibitors in patients with moderate severity haemophilia A
British Journal of Haematology 1996: 93:(Suppl.1), 60

198) Ollier W, Pepper L, Cumming A, Preston FE, Hill FGH, Colvin BT, Ludlam CA
HLA Class II profile: a determinant of factor VIII inhibitor development in severe haemophilia A
British Journal of Haematology 1996: 93:(Suppl.1), 60

199) Beauchamp NJ, Daly ME, Cooper PC, Makris M, Preston FE, Peake IR
The molecular basis of protein S deficiency in four unrelated kindred
British Journal of Haematology 1996: 93:(Suppl.1) 5

200) Preston FE, Walker ID, Rosendaal FR, Briet E
Increased fetal loss in women with thrombophilia
British Journal of Haematology 1996: 93:(Suppl.1) 5

201) Makris M, Phillips WS, Greaves M, Kitchen S, Rosendaal FR, Preston FE
Emergency oral anticoagulant reversal. The necessity for prothrombin complex concentrates
British Journal of Haematology 1996: 93:(Suppl.1) 6

202) Makris M, Preston FE
Coagulation activation following infusions of a factor XI concentrate
British Journal of Haematology 1996: 93:(Suppl.1) 10

203) Jennings I, Woods TAL, Kitchen S, Preston FE
Influence of method on quantitative and qualitative measurement of von Willebrand factor
British Journal of Haematology 1996: 93:(Suppl.1) 10

204) Ford I, Douglas CWI, Preston FE
The vitronectin receptor and platelet aggregation by streptococcus Sanguis
British Journal of Haematology 1996: 93:(Suppl.1) 25

205) Hough RE, Makris M, Preston FE
Pregnancy in women with thrombophilia: Incidence of thrombosis and pregnancy outcome
British Journal of Haematology 1996: 93:(Suppl.1) 29

206) Jennings I, Woods TAL, Kitchen S, Preston FE
UK NEQAS for Blood Coagulation: Detection of the lupus anticoagulant
British Journal of Haematology 1996: 93:(Suppl.1) 33

207) Jennings I, Woods TAL, Kitchen S, Preston FE
Agreement between laboratories in thrombophilia screening. Results from UK NEQAS for Blood Coagulation
British Journal of Haematology 1996: 93:(Suppl.1) 33

208) Makris M, Preston FE, Rice KM, Rosendaal F, Underwood JCE, Triger DR
Factors influencing the development of cirrhosis and liver failure in patients with congenital bleeding disorders and chronic hepatitis C
British Journal of Haematology 1996: 93:(Suppl.1) 50

209) Kitchen S, Jennings I, Woods TAL, Preston FE
Plasma calibrants for standardisation of coagulometer determined INR's. A NEQAS supplementary exercise
British Journal of Haematology 1996: 93:(Suppl.1) 91

210) Kitchen S, Preston FE
Monitoring oral anticoagulant therapy with the TAS near patient test system: Comparison with conventional thromboplastins
British Journal of Haematology 1996: 93:(Suppl.1) 91

211) Kitchen S, Jennings I, Woods TAL, Preston FE
Local calibration of coagulometers for INR determination: Results of a NEQAS supplementary exercise
British Journal of Haematology 1996: 93:(Suppl.1) 91

1997

212) Makris M, Rosendaal FR, Preston FE
Familial thrombophilia: genetic risk factors and management
Journal of Internal Medicine 1997: 242 (Suppl.740) 9-15

LETTERS

1) F E Preston, C A Jackson, M Greaves, C J Stoddard
Inhibition and recovery of prostacyclin and thromboxane A2 after l w dose Aspirin
Thrombosis and Haemostasis; 1981; 46; 25

2) F E Preston
The role of platelets in thromboembolic disease
British Journal of Haematology; 1981; 49; 143-144

3) F E Preston
Essential thrombocythaemia
The Lancet; 1982; i; 1021

4) M Greaves, F E Preston
Prostacyclin and thromboxane A2
British Medical Journal; 1982; 284; 419

5) F E Preston, D R Triger, J C E Underwood
Blood product concentrates and chronic liver disease
The Lancet; 1982; i; 565

6) F E Preston, M Greaves, A J M Boulton, J D Ward
Improved diabetic control and platelet reactivity
The Lancet; 1985; i; (Letter)

7) F E Preston, C R M Hay, M S Dewar, M Greaves, D R Triger
Non-A, Non-B Hepatitis and heat treated factor VIII concentrates
The Lancet; 1985; ii; 213

8) C R M Hay, F E Preston, D R Triger, J C E Underwood
Progressive liver disease in haemophilia; an understated problem?
The Lancet; 1985; i; 1495-1498

9) P B A Kernoff, E J Miller, G F Savidge, S J Machin, M S Dewar,
F E Preston
Wet heating for safer factor VIII concentrate?
The Lancet; 1985; ii; 721

10) C R M Hay, F E Preston, D R Triger, J C E Underwood
Liver disease in haemophilia
The Lancet; 1985; ii; 1187

11) L A Brawn, M A Parsons, P Allamby, L E Ramsay, F E Preston
Massive cardiac disease in a patient with Waldenstrom's macroglobulinaemia
Journal of Clinical Pathology; 1988; 41; 475-476

12) M Makris, F E Preston, D R Triger, J C E Underwood, Q L Choo, G Kuo, M M Houghton
Detection by PCR of hepatitis C virus in factor VIII concentrates
Lancet 1990, 335, 1117

13) F E Preston, M Makris, D R Triger, J C E Underwood
Prevention of hepatitis C virus infection in haemophiliacs
Lancet 1990, 336, 63

14) Makris M, Preston FE
Does HIV infection accelerate malignant transformation of pre-malignant states?
British Journal of Haematology 1992; 49: 220 (Letter)

15) Kitchen S, Preston FE
Factor VII clotting assays: Influence of thromboplastin
Thrombosis and Haemostasis 1993; 70(4): 720 (Letter)

16) Beauchamp NJ, Daly ME, Cooper PC, Preston FE, Peake IR
Rapid two stage PCR for detecting factor V G1691A mutation
Lancet 1994; 344: 694

17) Nesbitt IM, Goodeve AC, Preston FE and Peake IR
Von Willebrand factor/factor VIII binding is not affected by the Arg89Gln polymorphism in von Willebrand factor
Thrombosis and Haemostasis 1996; 76(5); 820-821

PUBLISHED PROCEEDINGS

1) W R Timperley, F E Preston, J D Ward
Intravascular coagulation in the nervous system
VIIth International Congress of Neuropathology
Excerpta Medica-Amsterdam; 1975; 581-584

2) F E Preston, W R Timperley, B C O'Malley, J D Ward
Intravascular thrombosis and platelet function in diabetic neuropathy
Proceedings of the Vth Congress of International Society of Thrombosis and Haemostasis; Paris; 1975 (Abstract)

3) D L Douglas, T Cundy, J A Kanis, F E Preston, R G G Russell, S Woodhead
Inhibitory action of dichlorothylene diphosphonate (Cl2MDP) on bone resorption in man
Proceedings 3rd Symposium CEMO on Bone and Tumours; Basle; Switzerland November 1979; A Donath & B Courvoisier (Eds); Editions Medicine et Hygiene, Geneve; 1980; 161-165

4) J A Kanis, A D Paterson, B J Beard, D L Douglas, F E Preston, R G G Russell
The use of Diphosphonates for increased bone resorption in malignant disease
International Congress Series no 589; Current Advances in Skeletogenesis
Proceedings of the 5th Worksop; Kiryat-Anavim; Israel; 1982; 498-503

5) F E Preston, R M Bertina, C Kluft, C Forbes, G D O Lowe, S L Blamey, R G Malia
Enhancement of Protein C by Stanozolol in healthy adults and in surgical patients.
Clinical Aspects of Fibrinolysis and Thrombolysis; J Jesperson, C Kluft, O Korsgaard (Eds); South Jutland Press; Esbjerg; Denmark; 1983; 397-398

6) M Greaves, F E Preston, C Kluft, R M Bertina, R G Malia, M Simms
A comparative study of the influence of intramuscular stanozolol and subcutaneous heparin on coagulation and fibrinolysis following abdominal surgery
Progress in Fibrinolysis; Davidson, Donati, Coccheri (Eds)
Churchill Livingstone; 1985; 7; 105-107

7) J H Verheijen, D C Rijken, G T G Chang, F E Preston, C Kluft
Decreased level of rapid tissue-type plasminogen activator inhibition by oral administration of Stanozolol
Progress in Fibrinolysis; Davidson, Donati, Coccheri (Eds)
Churchill Livingstone; 1985; 102-104

8) S Kitchen, R G Malia, F E Preston
Automation and haemostasis; The Automated coagulation Laboratory (ACL) Centrifugal Analyser
Proceedings of the 2nd International Symposium on Standardisation and Quality Control of Coagulation Tests
Rome, Italy; 1989

9) Schwarz H P, Varadi K, Moritz B, Lang H, Bauer K, Preston FE, Peake IR, Rivard GE
A chromogenic assay for activated protein C (APC) resistance
Presented at the 36th Annual meeting of the American Society of Haematology, 2-6.12.94, Nashville, Tennessee, USA

CHAPTERS

1) A Davies-Jones, F E Preston, W R Timperley
Neurological Complications in Clinical Haematology
Blackwell Scientific Publications 1980

2) M Greaves, F E Preston, C A Jackson, A J Boulton, J D Ward
Clinical Aspects of Fibrinolysis and Thrombolysis
Changes in Fibrinolytic Activity and Fibrinogen Concentration in Response to improved Glycaemic Control in Diabetic Subjects
J Jesperson, C Kluft, O Korsgaard (Chapter Eds), South Jutland Press, Esbjerg, Denmark, (1983), 345-356

3) M Greaves, F E Preston
Haemostatic abnormalities in diabetics
Diabetes and Heart Disease
R J Jarrett (Chapter Ed) Elsevier, (1984) 2: 47-80

4) M Greaves, F E Preston
The laboratory investigation of acquired and congenital platelet disorders
Blood Coagulation and Haemostasis
J M Thompson (Chapter Ed) Churchill Livingstone, 3rd Edition, (1985)

5) M Greaves, F E Preston, C Kluft, R M Bertina, R G Malia, M Simms
A comparative study of the influence of intramuscular stanozolol and subcutaneous heparin in coagulation and fibrinolysis following abdominal surgery.
Progress In Fibrinolysis
Davidson, Donati Coccheri (Chapter Eds), (1985), 7: 105-107

6) G Dolan, J Ball and F E Preston
Protein C and Protein S
Clinical Haematology, Publishers Balliere Tindall, London
1989, Vol 2; No 4; The Molecular Biology of Coagulation; 999-1042

7) P C Cooper, F E Preston
Euglobulin clot lysis time
ECAT Assay Procedures, A Manual of Laboratory Techniques
Editors: J Jespersen, R M Bertina, and F Havercate
Kluwer Academic Publishers, 1992, 15; 125-129

8) F E Preston, E Briet
Familial thrombophilia
Recent Advances in Haematology 7
Editors: Brenner and Hoffbrand
Publishers: Churchill Livingstone, London, 1993, 12, 217-239

9) F E Preston and M Greaves
Laboratory Diagnosis of Hereditary Thrombophilia
Hypercoagulable States; Biological Aspects and Clinical Management
Editors: J Seghatchian and M M Samama
Publishers: CRC Press, Inc. Florida, USA, 1995.

10) M Greaves and F E Preston
Pathogenesis of thrombosis: antithrombotic therapy
In Postgraduate Haematology (4th edition)
Editors: Hoffbrand, Lewis, and Tuddenham
Publishers: Butterworth Heinemann, Oxford 1998.

11) Advances in Laboratory Methods: General Haematology
Editors: O W van Assendelft, R M Rowan, and F E Preston
Publishers: Butterworth Heinemann, Oxford 1998.

12) TAL Woods, S Kitchen, F E Preston
Quality Assessment of Haemostatic Assays and External Quality Assessment Schemes
In Haemostasis and Thrombosis: A Manual of Laboratory Techniques
Editors: J Jespersen, R M Bertina and F Haverkate
Publishers: Kluwer Academic Publishers, The Netherlands 1998.